

IMPORTANCE OF INFECTIOUS PANCREATIC NECROSIS VIRUS
IN STRIPED BASS, Morone saxatilis

By

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Infectious pancreatic necrosis virus (IPNV), a pathogen for Atlantic menhaden (Brevoortia tyrannus), was isolated recently from striped bass fry (Morone saxatilis) in a hatchery on the Chesapeake Bay (MD). The major goal of this study was to investigate the effects of IPNV infection in striped bass.

No clinical or histopathological signs of disease were observed in striped bass exposed to IPNV by immersion or intraperitoneal injection. This was true even in IPNV-exposed striped bass that were subjected to an abrupt drop of pH or a temperature change. Chronic IPNV infection was not detected in striped bass challenged with waterborne virus; however, striped bass that consumed or were inoculated with IPNV contained infectious virus for at least eight months, despite the presence of circulating virus-neutralizing antibody.

Striped bass develop virus-neutralizing antibody by seven days after IPNV inoculation. This humoral response could be depressed by exogenous corticosteroids. Striped bass did not exhibit an anamnestic response, but did have increased antibody titers after a second intraperitoneal injection with IPNV.

A few striped bass caught in the Chesapeake Bay had IPNV-neutralizing antibody, although no IPNV was isolated from these fish. The source of exposure for the striped bass is not known. Neutralization kinetics and patterns of viral polypeptides in SDS-polyacrylamide gel electrophoresis demonstrated that the IPNV isolates from striped bass and menhaden are closely related to each other and to the salmonid isolate VR-299.

Virus-infected striped bass transmitted IPNV to brook trout; therefore, striped bass should be assayed for IPNV prior to their introduction into IPNV-free areas. Detection of IPNV-carriers was improved if striped bass received steroids prior to assay. A population of striped bass from which IPNV has been isolated need not be destroyed since striped bass appear to be resistant to IPNV-induced disease.

CHAPTER ONE INTRODUCTION

Infectious pancreatic necrosis virus (IPNV), a significant pathogen for salmonids (Wolf et al., 1960), has been recovered from many fish species (Hill, 1982; Ahne, 1985). Recently IPNV was isolated from striped bass (Morone saxatilis) fry in a hatchery on the Chesapeake Bay (Schutz et al., 1984). Efforts to raise striped bass in hatcheries have increased (Schutz et al., 1984), partly in response to declining populations of striped bass on the east coast of the United States (Goodyear et al., 1985). Because IPNV can devastate hatchery populations of trout (Wolf et al., 1960), this investigation was initiated to study the impact of IPNV infection on striped bass.

Background

Early in this century, many North American trout hatcheries experienced annual epizootics that resulted in massive losses of young fry, affecting the fastest growing individuals first (M'Gonigle, 1941). In 1955, Wood et al. described microscopic lesions of pancreatic necrosis in affected trout fry and also demonstrated that the condition could be transmitted to fish located downstream from affected fish. Wood et al. (1955) named the disease

infectious pancreatic necrosis (IPN). Although Wood et al. (1955) speculated that the pathogenic agent was a virus, the viral nature was not demonstrated until 1960 by Wolf et al. Wolf and coworkers (1960) used filtered homogenates of clinically affected trout to challenge fish and cell cultures. Significant numbers of exposed fish died and cytopathic effects (CPE) were apparent in inoculated fish cell cultures. Electron microscopy revealed that IPNV is a naked, icosahedral virus, between 55 - 75 μm in diameter (Moss & Gravell, 1969; Cohen & Scherrer, 1972; Kelly & Loh, 1972). The virus may exist also as tubular particles (Moss & Gravell, 1969; Ozel & Gelderblom, 1985). The genome of IPNV consists of two segments of double-stranded ribonucleic acid (RNA); the molecular weight of one segment is 2.5×10^6 and the other is 2.3×10^6 (Dobos, 1976; Macdonald & Yamamoto, 1977). The latter segment encodes the largest viral associated polypeptide, and the former encodes the other proteins (Macdonald & Dobos, 1981; Mertens & Dobos, 1982).

The viral polypeptides of IPNV fall into three general molecular weight classes--low, medium, and high (Cohen et al., 1973; Dobos & Rowe, 1977; Chang et al., 1978). The high molecular weight ($90 - 105 \times 10^3$) polypeptide corresponds to the polymerase, the enzyme that catalyzes the synthesis of messenger RNA (Macdonald & Dobos, 1981; Stephens & Hetrick, 1983). The capsid protein, the major component, is of medium weight ($50 - 57 \times 10^3$), and the

internal proteins are of low molecular weight ($27 - 31 \times 10^3$) (Macdonald & Dobos, 1981).

Because the viral genome consists of two segments of double stranded RNA, Dobos et al. (1979) proposed that IPNV be classified a birnavirus. Included in this proposed group are infectious bursal disease virus (IBDV), found in young chickens (Nick et al., 1976); Drosophila X virus, isolated from fruit flies (Teninges et al., 1979); and Tellina virus and oyster virus, isolated from bivalve molluscs (Hill, 1976; Underwood et al., 1977). Although these viruses are similar morphologically and biochemically, they can be distinguished serologically and by comparison of the virion-associated proteins (Dobos et al., 1979). None of the birnaviruses, except IPNV, have been demonstrated to be pathogenic for fish.

In young trout IPNV infection may be manifested either as acute death or by fish that exhibit brief episodes of violent spinning after which the fish sink to the bottom of the tank (Wolf, 1981). Death usually occurs within 1 - 2 days after onset of clinical signs. Upon necropsy, dead or moribund fish may have multiple petechial hemorrhages on the internal organs. Wolf (1981) considers the finding of a clear to cloudy gelatinous material in the stomach and anterior intestine to be pathognomonic for IPNV in young trout. Histological lesions include necrosis of pancreatic acini (Lightner & Post, 1969; Swanson et al., 1982) and

frequently, acute catarrhal enteritis (McKnight & Roberts, 1976). Considerable portions of the pancreas become fibrotic in trout that survive IPNV infection (McKnight & Roberts, 1976; Swanson et al., 1982).

The exact mechanisms by which IPNV causes death in infected fish are not known (Hill, 1982). Correlation between virus titers and severity of disease has been reported (Okamoto et al., 1984). Virus titers progressively rise in trout fry following challenge with IPNV and the highest titers of virus are recovered from fish that have died (Okamoto et al., 1984). Swanson and Gillespie (1982) speculated that key events occurred within the first few days following viral challenge. Using experimentally infected Atlantic salmon (Salmo salar), Swanson and Gillespie (1982) noted that peak viremia occurred at day two. Swanson and Gillespie (1982) stated that the Atlantic salmon, unlike trout, are successful, by some unexplained mechanism, in preventing further increases in viral titers, thus preventing IPNV-induced mortality.

For reasons yet to be determined, by six months of age trout lose their susceptibility to IPNV-induced mortality (Frantsi & Savan, 1971; Wolf, 1972). In addition, trout species differ in their susceptibility to IPNV-induced mortality (Hill, 1982; Silim et al., 1982). The resistance may be mediated genetically. Wolf (1976) reported the development of IPNV-resistant trout strains, using selective breeding.

Many different factors have been described that affect the outcome of IPNV infection on trout. Frantsi and Savan (1971) demonstrated that water temperature affects the number of deaths associated with IPNV. The authors found fewest deaths in viral-exposed trout fry kept at 4.5°C, most at 10°C, and an intermediate number of deaths in fry held at 15°C. Also, as mentioned earlier, the age at which fish are exposed to IPNV affects IPNV-induced disease (Dorson & Torchy, 1981). Young fish (less than six months) are more susceptible to IPNV-induced mortality, but older trout do become subclinically infected with IPNV (Frantsi & Savan, 1971).

Stress was also found to influence IPNV infection, especially in trout that survived early exposure but continue to be infected. Frantsi and Savan (1971) found an increase in IPNV isolation from trout survivors after an episode of mild stress induced by low oxygen. McKnight and Roberts (1976) reported 10 - 20% mortality in IPNV-carrier rainbow trout (ages 6 to 11 months) at 72 hours following a stressful event such as handling, transport, overcrowding, or low oxygen. Higher IPNV titers were obtained from stressed fish compared to titers from non-stressed fish (McKnight & Roberts, 1976).

It is not known how IPNV persists in infected trout. Normally IPNV multiplies intracytoplasmically and is released by viral-induced cytolysis (Malsberger & Cerini,

1963; Argot & Malsberger, 1972). Defective interfering particles are produced within cells, but do not cause cell rupture (Nicholson & Dunn, 1974; Macdonald, 1978). Therefore, this may be a mechanism by which IPNV persists in carriers (Nicholson & Dexter, 1975; Hedrick et al., 1978; Macdonald & Kennedy, 1979). Other researchers have proposed a relationship between levels of virus-neutralizing antibodies and titers of IPNV; i. e. fish with high levels of IPNV-neutralizing antibody would have lower titers of IPNV (Yamamoto 1975a, 1975b). However, there is no correlation between the tissue levels of virus and antibody titers in IPNV-carrier trout (Reno, 1976; Reno et al., 1978). Another mechanism by which IPNV persists may be due to an IPNV-induced decrease in the mitogenic responsiveness of lymphocytes and macrophages (Knott & Munro, 1986).

Trout survivors present after an episode of IPNV disease continue to contain, and periodically shed, IPNV (Wolf et al., 1968; Billi & Wolf, 1969; Yamamoto & Kilistoff, 1979). Fish located downstream from the effluent of an IPNV-infected hatchery can become infected with IPNV (Sonstegard et al., 1972). The virus can be spread by other animal vectors. Gulls, chickens, and mink, after being fed IPNV-infected fish, transiently shed virus in their feces (Eskildsen & Jorgensen, 1973; Sonstegard & McDermott, 1972). Once shed, IPNV can survive for weeks in dried areas (Wolf, 1966; Ahne, 1982), or for months in aqueous environments (Desautels &

MacKelvie, 1975; Baudouy & Castric, 1977; Wedemeyer et al., 1978).

Another means by which IPNV may be spread is by the transport of eggs taken from IPNV-infected stocks (Hill, 1982). Although egg-associated transmission of IPNV was suggested as early as 1959 (Snieszko et al.), and was documented in 1963 (Wolf et al.), transport of eggs from IPNV-infected stocks continued, perhaps resulting in the international spread of the virus (Sano, 1971).

Disease outbreaks associated with IPNV have been reported around the world. Although the virus always is morphologically similar, IPNV has several serotypes (Wolf & Quimby, 1971; McMichael et al., 1975). Different serotypes signify that an antibody generated against IPNV isolated from one disease outbreak may, or may not, react with IPNV recovered from a different location or disease episode. The virus has three major serotype groups: (1) most North American IPNV isolates (Buhl, Reno, Powder Mill, West Buxton, Cascade Locks, VR-299); (2) isolates from Denmark and France (d'Honninethun, Bonnamy, Sp); and (3) IPNV from Denmark and Japan (Ab, EEV) (Okamoto et al., 1983). The exact placement of IPNV isolates varies somewhat between authors (Macdonald & Gower, 1981; Ishiguro et al., 1984). The differences probably are related to the variation of methods and antisera used to determine serotypes (Nicholson & Pochebit, 1981), and to variations in sensitivity of the isolates to neutralization (Macdonald & Gower, 1981).

Isolates of IPNV differ somewhat in their stability during storage and freeze-thaw cycles (Wolf & Quimby, 1971; Lientz & Springer, 1973; McMichael et al., 1975). However, despite the differences in serotype and variation in storage stability, IPNV isolates induce similar clinical signs in challenged trout (Wolf & Quimby, 1971; Silim et al., 1982). The virus has been isolated from many clinically normal non-salmonid fishes including white sucker, Catostomus commersoni (Sonstegard et al., 1972); perch, Perca fluviatilis (Munro et al., 1976); European eel, Anguilla anguilla (Castric & Chastel, 1980); bream, Abramis brama (Adair & Ferguson, 1981); Atlantic silverside, Menidia menidia (McAllister et al., 1984); tilapia, Tilapia mossambica (Chen et al., 1985); and goldfish, Carassius auratus (Hedrick et al., 1985). In addition, IPNV has been recovered from moribund nonsalmonids, including northern pike (Esox lucius) (Ahne, 1978), sea bass (Dicentrarchus labrax) (Bonami et al., 1983), and southern flounder (Paralichthys lethostigma) (McAllister et al., 1983). However, the pathogenicity of IPNV has not been demonstrated for these species. Experimental transmission studies using the pike isolate did not induce viral disease in either pike or rainbow trout (Ahne, 1978), and similar avirulence was observed for the flounder isolate in both flounder and brook trout (McAllister et al., 1983).

The lack of demonstrable pathogenicity of IPNV has also been reported for other nonsalmonids. Experimental IPNV infection of various marine species did not cause clinical disease, although virus multiplication probably occurred in the french grunt, Haemulon flavolineatum (Moewus-Kobb, 1965). Vertical transmission of IPNV was demonstrated in experimentally inoculated zebra fish, Brachydanio rerio (Seeley et al., 1977), although no disease was detected in the offspring.

In contrast, IPNV has been shown to be pathogenic for three nonsalmonid species. An IPNV isolate has been demonstrated to induce high mortality and brachionephritis in Japanese eels, Anguilla japonica (Sano et al., 1981). In yellowtail, Seriola quinqueradiata, experimental inoculation with IPNV resulted in high mortality in fingerlings that developed ascites and hepatic hemorrhage (Sorimachi & Hara, 1985). Atlantic menhaden, Brevoortia tyrannus, injected with IPNV developed dark coloration and hemorrhage at fin bases, and began swimming in circles prior to death 3 - 5 days post inoculation (Sterhens et al., 1980). Virus was reisolated from the brain, kidney, spleen, liver, blood and gonadal tissue from menhaden that died.

In 1984, Schutz et al. reported the isolation of IPNV from striped bass fry in a hatchery operated by the Baltimore Gas and Electric, Co. Virus was recovered from fry exhibiting erratic swimming behavior and high

mortality. Histological examination of moribund fry revealed areas of necrosis in the epidermis. The virus was isolated from kidneys taken from surviving striped bass at three and six months following the original IPNV isolation. Inflammation around pancreatic acini was observed in histological sections taken from the survivors at three months. This constellation of findings resembles that found in salmonids in which IPNV causes death in young fry and histopathological lesions in pancreatic acini of infected fish. Thus, it was hypothesized that IPNV may cause mortality in striped bass fry (Schutz et al., 1984).

Striped bass traditionally have been important both as commercial and recreational fish (Morgan & Rasin, 1981); however, the Chesapeake Bay stocks of striped bass have been declining (Goodyear et al., 1985). The reasons for this decline are not known, although many possibilities have been suggested. These include loss of appropriate habitat (Kerhehan et al., 1981), overfishing (Coutant, 1985), starvation of fry (Eldridge et al., 1981), pollution (Hall et al., 1984), and temperature and oxygen levels (Coutant, 1985). In addition, disease might be contributing to the decline. "Spinning disease" can be induced by IPNV in Atlantic menhaden (Stephens et al., 1980) and a disease episode was occurring in menhaden in the Chesapeake Bay at the time that IPNV was isolated from the moribund striped bass fry. Records kept by the

Maryland Department of Natural Resources indicated a correlation between large outbreaks of "spinning disease" in menhaden and poor year classes of striped bass in the Chesapeake Bay (Schutz et al., 1984).

Objectives

Research was initiated to investigate the importance of IPNV infection in striped bass. The points to be specifically addressed were (1) whether IPNV induced mortality in striped bass; (2) what histological lesions developed in striped bass exposed to IPNV; (3) the influence of age and strain of striped bass on IPNV virulence; (4) the effect of water temperature on IPNV-induced disease in striped bass; (5) the routes (both vertical and horizontal) by which IPNV is transmitted in striped bass; (6) the influence of stress, including abrupt environmental changes and exogenous corticosteroids, on IPNV infection in striped bass; (7) the humoral response of striped bass to IPNV; (8) comparison of the striped bass isolate of IPNV with a menhaden IPNV isolate and the standard North American salmonid isolate (VR-299); and (9) the effects of sample handling procedures on viral infectivity.

CHAPTER TWO MATERIALS AND METHODS

Cell Cultures and Virus Isolates

Cell Cultures

Chinook salmon embryo (CHSE-214) cells were grown at 18°C in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (EMEM-10). For cell transfers, confluent monolayers were dispersed with 0.25% trypsin. For virus assays, cells were suspended in EMEM-10 containing antibiotics: 200 IU/ml penicillin and 200 ug/ml streptomycin (PS). Cells were seeded into eight-well culture plates and incubated at 18°C in ambient air plus 2% carbon dioxide (CO₂) until monolayers were confluent.

Isolates of IPNV

The striped bass isolate of IPNV (IPNV-Sb) that was originally isolated from moribund striped bass fry (Schutz et al., 1984), was used for all experiments, except where noted. The virus was passaged twice in CHSE-214 cells and aliquots were stored at -70°C.

Three other isolates of IPNV, the standard North American isolate (VR-299), an isolate from Atlantic menhaden (IPNV-M), and a European isolate (IPNV-Ab), were handled as described for IPNV-Sb. Before use, aliquots of virus were thawed and diluted in phosphate buffered saline (pH 7.2; PBS).

Cultivation and Assays of IPNV

Preparation of Virus Stocks

Confluent monolayers of CHSE-214 cells grown in 75 cm² flasks, were drained of medium and inoculated with IPNV (< 0.01 plaque forming units [pfu] of IPNV per cell). Following an one hour adsorption period at 15°C (with gentle agitation every 15 minutes), EMEM-10 was added to the IPNV-inoculated cells. The virus-exposed cells were incubated at 15°C until the monolayers showed extensive cytopathic effects (CPE) (usually 2 - 3 days). Cells and culture fluid were harvested and centrifuged at 1500 x g for 15 minutes at 4°C. The supernatant liquid was stored in 1 ml aliquots at -70°C. The infectivity of stock virus was determined by plaque assay as described below.

Virus Infectivity Plaque Assay

A modification of a virus infectivity assay (Moss & Gravel, 1969) was used to determine virus titers. Aliquots (0.1 ml) of each sample dilution were inoculated onto duplicate wells of drained CHSE-214 monolayers. The inoculated monolayers were incubated for 1 hour at 19°C to allow virus adsorption and then were overlaid with EMEM containing 2% normal calf serum, 0.16 M Tris buffer and PS (EMEM-2), plus 1% agarose. A second overlay, 2 ml of EMEM-2 (without agarose), was added. Plates were incubated at 18°C in ambient air plus 2% CO₂ until cytopathic effects (CPE) were noted. Cell sheets were fixed with 30% formalin,

and stained with 1% crystal violet in ethanol. Plaques were counted and infectivity titer was calculated as pfu per ml or pfu per g of tissue.

Virus Infectivity Assay

The simultaneous seeding method (McDaniel, 1979) was used to detect IPNV in striped bass fry and striped bass sex products. An aliquot (0.05 ml) of each sample dilution was added to each of four wells of a 96-well tissue culture plate and then 0.1 ml of CHSE-214 cells (about 2×10^5 cells/ml) was added to each well. Plates were incubated in ambient air at 18°C. If no CPE was observed by 5 days, the sample was harvested from the wells and inoculated with fresh cells (blind-passaged). If no CPE was noted after 5 additional days, the sample was considered to be negative for IPNV.

Characterization of IPNV-Sb

Purification of IPNV Isolates

Three isolates of IPNV, the striped bass isolate (IPNV-Sb), the menhaden isolate (IPNV-M), and the North American isolate (VR-299), were each purified using a modification of a procedure previously described by Chang et al. (1978). Confluent monolayers of CHSE-214 cells were inoculated with virus (< 0.01 pfu/cell). The virus was allowed to adsorb for 1 hour at 15°C, then EMEM-2 was added. After 48 hours incubation at 15°C, the cell sheets

showed extensive CPE. The cells and culture fluids were centrifuged at $7000 \times g$ for 20 minutes at 4°C . The cell pellet was resuspended in 5 ml of buffer made up of 0.01 M Tris, 0.01 M sodium chloride, and 0.001 M disodium ethylenediamine tetraacetate (TNE; pH 7.5). A equal volume (5 ml) of trichlorotrifluoroethane (Freon) was added and the solution was homogenized for two minutes at high speed. The homogenate was centrifuged at $4500 \times g$ for 15 minutes at 4°C . The top layer of TNE was removed and stored at 4°C . An additional 5 ml of TNE were added to the Freon-cell mixture. This solution was homogenized and centrifuged as described above. The TNE layer was combined with the first freon-extract. The original cell supernatant fluid was adjusted to contain 6% (wt/v) polyethylene glycol (M.W. 20,000), and 2.2% (wt/v) sodium chloride. This mixture was stirred for 3 hours at 4°C . The solution was centrifuged at $9000 \times g$ for 1 hour at 4°C . The supernatant liquid was discarded and the pellet was resuspended in the TNE layer (5 - 8 ml) from the freon extraction. This suspension was gently layered over a sucrose or cesium chloride (CsCl) gradient.

For IPNV samples that were used to inoculate rabbits, the crude virus preparation was purified on a linear sucrose (10 - 50%) density gradient in Ultra-clear centrifuge tubes (25 x 76 mm) that were centrifuged at $97,000 \times g$ for 45 minutes at 4°C . The virus band was withdrawn by side tube puncture using a 20 gauge needle and

5 ml syringe. The virus band was diluted in TNE and centrifuged at $83,000 \times g$ for 40 minutes at 4°C . The viral pellet was resuspended in 1 ml TNE and stored at -70°C . Protein content was measured by the method described below.

For IPNV samples that were analyzed for virus specific proteins, the crude virus preparation was purified on a linear CsCl (20 to 40%) gradient in cellulose nitrate centrifuge tubes (5/8" x 4") that were centrifuged at $115,000 \times g$ for 16 hours at 4°C . The virus band was removed by side tube puncture with a 22 gauge needle and 5 ml syringe, diluted in TNE, and layered over a second CsCl gradient. The band containing pure virus was removed, dialyzed against TNE, and concentrated to 1 ml using membrane microconcentrators. Protein concentration was determined as described below and infectivity was quantified by the plaque assay.

Determination of Protein Concentration

The Lowry method (Lowry et al., 1951), as modified by Garvey et al. (1977), was used to determine the protein concentration of the purified IPNV isolates (IPNV-Sb, IPNV-M and VR-299). Bovine albumin was diluted (1 to 0.01 mg/ml) in phosphate buffered saline for protein standards. One milliliter of a solution containing 2% sodium carbonate, 0.02% cupric sulfate in 0.1 N sodium hydroxide was added to 0.2 ml of each unknown and standard sample. The samples were mixed, incubated for 10 minutes at 25°C ,

and then 0.1 ml of 1 N phenol reagent was added. After 1 hour incubation at 25°C, the optical density of each sample at 660 nm was determined by spectrophotometry. All samples were assayed in duplicate. The mean optical density of each standard was plotted against the protein concentration. The protein concentration of the unknown samples were calculated by interpolation from the standard line.

Electrophoresis of Viral Polypeptides

Comparison of the structural proteins of three IPNV isolates (IPNV-Sb, IPNV-M, and VR-299) was performed by examination of the banding pattern of the viral proteins in discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Tris-glycine buffer method described by Laemmli (1971) was used. A 10% resolving gel was prepared by combining 13.3 ml of 30% acrylamide, 0.4 ml of 10% sodium dodecyl sulfate, 10.0 ml of 18.5% tris-HCl (pH 8.8), and 16.2 ml of distilled water. The solution was degassed under vacuum for 15 to 30 minutes and 0.1 ml of 10% ammonium persulfate and 0.02 ml of N,N,N',N' tetramethyl ethylenediamine (TEMED) were added. The mixture was gently swirled and poured into the gel mold. After polymerization (20 to 30 minutes), the gel was rinsed with distilled water. The gel was overlaid with a buffer containing 2.5 ml of 18.5% Tris-HCl (pH 8.8), 0.1 ml 10% SDS and 7.4 ml distilled water, and allowed to stand

overnight. The 4% acrylamide stacking gel was made by combining 1.3 ml of 30% acrylamide, 2.5 ml of 6% Tris-HCl (pH 6.8), 0.1 ml of 10% SDS and 6.1 ml of distilled water. This mixture was degassed as above; then 0.05 ml of 10% ammonium persulfate and 0.005 ml of TEMED were added. The solution was gently swirled and poured on top of the resolving gel. After 20 minutes, the gel was rinsed with distilled water. Total gel size was 1.5 mm x 14 x 16 cm. Ten ug of each IPNV isolate were mixed with a solution that contained 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue, and 1.5% Tris-HCl. The sample solution was heated to 100°C for three minutes, cooled to 4°C, and loaded onto the gel. Running buffer (3.0 g Tris, 14.4 g aminoacetic glycine and 1 g SDS in one liter of distilled water) was placed in the upper and lower chambers. A direct current of fifteen mAmps was applied until the bromphenol blue dye line passed through the stacking gel. The current was then raised to 25 mAmps and held constant until the dye line was 1 cm from the bottom of the resolving gel. The gel was removed, put into a solution of 50% methanol and 10% acetic acid for one hour, and stained overnight in a solution of 0.01% coomassie blue, 25% methanol and 10% acetic acid. The gel was destained using a solution of 25% methanol and 10% acetic acid.

The molecular weights of the IPNV structural proteins were determined by comparison to the electrophoretic

mobility of proteins of known molecular weight run in the same gel. The following molecular weight markers were used: phosphorylase B (97,400), bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,100). The relative mobility (D_f) of each polypeptide band was determined by dividing the distance traveled by each protein band by the distance traveled by the dye front. The logarithm₁₀ of the molecular weight of the marker proteins were plotted against the D_f . The molecular weight of each viral protein was determined from this standard line.

Production of Antiserum to IPNV-Sb

Antibody to the striped bass isolate of IPNV (IPNV-Sb) was prepared in New Zealand White rabbits. Purified IPNV-Sb was diluted to give 1 mg/ml in PBS. Rabbits were injected intravenously with 0.3 ml of this preparation. The remaining 0.7 ml was mixed with an equal volume of Freund's incomplete adjuvant. Half of this mixture (0.7 ml) was injected intramuscularly; the remaining 0.7 ml was injected subcutaneously into two different sites. The same procedure was repeated at two week intervals, for one month. Two weeks after the third boost, the rabbit was exsanguinated. The blood was held overnight at 4°C and centrifuged at 1500 x g at 4°C for 20 minutes. Serum was

collected, heated at 56°C for 30 minutes to inactivate complement, filtered using a membrane filter (0.45 micron pore size), and stored in 1 ml aliquots at -70°C.

Neutralization Kinetics

Antigenic differences in closely related viral isolates can be determined from analysis of the patterns and rates of neutralization (neutralization kinetics) of virus isolates in reactions with homologous and heterologous antisera. A modification of the procedure described by Macdonald and Gower (1981) was used to determine the antigenic relationships between three IPNV isolates (IPNV-Sb, IPNV-M, VR-299). Rabbit antisera to IPNV-M and VR-299 were available at the U. S. Fish and Wildlife Service, National Fish Health Research Laboratory, Kearneysville, WV. Antibody to IPNV-Sb was prepared in rabbits as described above. Each antibody was diluted to a concentration that neutralized 50% more homologous virus at 5 minutes than at 0.25 minutes after combination of antibody and virus. Each IPNV isolate (IPNV-Sb, IPNV-M, VR-299) was diluted in PBS to give a final concentration of 100 - 200 pfu/well as determined by plaque assay.

For each trial, antibody was assayed with its homologous and the two heterologous IPNV isolates. Equal volumes of antibody and IPNV were combined and incubated at 4°C. A 25 uL sample was removed at 0.25, 0.5, 1.0, 1.5, 2.0, 3, 4 and 5 minutes. The 25 ul sample was expelled

into 2.5 ml of PBS to stop the reaction and tested for residual infectivity using the plaque method. The mean plaque count of four replicate wells was calculated for each time point. The percent of residual infectivity was plotted against reaction time for each combination of IPNV isolate and antiserum. For the purpose of calculation of the rate of neutralization (K), K was assumed to be linear for the first 0.25 minute of the reaction and was determined by the formula $K = D/t \cdot 2.3 \log V_0 / V_t$, where D = the reciprocal of the dilution of antibody, t = 0.25 minute, V_0 = total virus and V_t = the number of virus plaques at 0.25 minute (Macdonald & Gower, 1981).

Sample Processing for IPNV Assays

Processing of Fish Tissues for IPNV Assay

Striped bass fry and striped bass sex products were processed using the following protocol prior to being assayed for IPNV. Five to 10 fry were washed twice in PBS and blotted on paper towels to remove excess water. Striped bass fry, or sex products, were added to 1 ml of PBS. The mixture was pulled into a 3 or 5 ml syringe through a 20 or 22 gauge needle, forcibly expelled several times to disrupt the tissues, and filtered using a membrane filter (0.45 micron pore size). Four ten-fold dilutions of each sample were assayed for IPNV using the simultaneous seeding method.

Fingerlings weighing less than 5 grams were processed as whole fish. Larger fish were dissected using sterilized instruments and the internal organs, blood and feces were assayed for virus. Samples were weighed and dissociated using a sterile pestle and alundum. The resultant paste was suspended 1:10 (wt/v) in PBS and centrifuged at 1500 g for 30 minutes at 4°C to sediment debris. Four serial dilutions of supernatant fluid was assayed for infectious virus using the plaque method.

Preparation of Striped Bass Blood for IPNV Assay

Striped bass blood samples were obtained from the caudal vein, by venipuncture using a 20 to 22 gauge needle or by severing the caudal peduncle. Blood was collected in heparinized microhematocrit capillary tubes. Within 2 hours of collection, blood samples were centrifuged and processed for virus assay as follows. The buffy coat (about 1 ul) was cut from the microhematocrit tube at the interface of the packed cells and plasma, placed into 0.5 ml sterile distilled water with PS, vigorously mixed, and incubated at 19°C for 1 hour. An additional 0.5 ml of PBS was then added to give about a 1:1000 (v/v) dilution of the buffy coat. An additional dilution (1:10) was made in PBS. The plasma was expelled into 1.0 ml PBS and a second dilution (1:10) made. These samples were immediately assayed for IPNV using the plaque assay.

Detection of Virus-Neutralizing Antibody

Fish Blood Preparation for Neutralization Assay

Fish blood samples were obtained from the caudal vein as described above and collected in heparinized microhematocrit capillary tubes or in plain tubes. Tubes were centrifuged and the supernatant fluid removed. Because preliminary assays indicated that normal striped bass blood neutralized IPNV at serum or plasma dilutions less than 1:100, all striped bass blood samples were assayed for virus-neutralizing antibodies at 1:100 or greater dilution. After centrifugation of the blood samples, 10 μ l of fish plasma/serum were added to 1.0 ml of PBS, heated at 45°C for 30 minutes to inactivate complement (Sakai, 1981), and stored at 4 or -20°C.

Virus-Neutralizing Antibody Assay

To detect circulating virus-neutralizing antibodies, serum or plasma samples are incubated with a known amount of virus, and then the residual infectivity determined. The following protocol was used for detection of IPNV-neutralizing antibody in striped bass. Equal volumes of fish serum or plasma sample and IPNV (1.6×10^3 pfu/ml) were mixed, and, with periodic gentle agitation, incubated at 19°C for one hour. Residual infectivity was determined by plaque assay. Total virus was determined by combining equal volumes of test virus and PBS and measuring virus infectivity. Blood samples were tested at the 1:100

dilution and were recorded as being positive for neutralizing activity if the sample neutralized 50% or more of total virus. Antibody titer was determined by testing serial dilutions of plasma against a constant number of virus and calculating the serum dilution that neutralized 50% of total virus (Reed & Muench, 1938). Unless otherwise indicated, fish samples were tested only against the striped bass isolate of IPNV (IPNV-Sb).

Fish

Striped bass fry from Maryland (Delmarva Ecological Laboratories, Elkton, MD) were held at the Baltimore Gas and Electric Company (MD) striped bass hatchery located on the Chesapeake Bay (MD). All other experimental fish were maintained at the U.S. Fish and Wildlife Service, National Fish Health Research Laboratory (Kearneysville, WV). Striped bass fry were obtained from Richmond Hill State Fish Hatchery (GA) and Richloam Fish Hatchery (FL). Striped bass fry were provided with brine shrimp (Artemia salina) nauplii as live food.

Striped bass fingerlings obtained from Harrison Lake National Fish Hatchery (VA) were maintained in 15 L tanks that received 4 L/min of 22°C spring water unless otherwise noted. Striped bass fingerlings were fed commercial salmon or trout food. Five-year-old striped bass obtained from Edenton National Fish Hatchery (NC) were kept in spring and reservoir water (12 - 25°C). Rainbow

(Salmo gairdneri), brook (Salvelinus fontinalis) and brown (Salmo trutta) trout were provided as forage.

Brook trout fingerlings were obtained from White Sulfur Springs National Fish Hatchery (WV) and were held in 12°C spring water and fed trout ration.

Virus Infection Studies

Waterborne IPNV Challenge of Striped Bass Fry

This series of virus challenge trials was conducted to determine if IPNV would induce significant mortality in striped bass. Several factors, including age at exposure, strain of striped bass, water temperature, and environmental stress, were investigated for their effect on striped bass exposed to a static IPNV-bath.

Striped bass fry from Florida and Georgia were divided into groups of 60 and held in 500 ml tissue culture bottles containing spring water (19°C). Florida fry were challenged with IPNV at 1, 3, 5, 7, 10 and 15 days post-hatch. Georgia fry were exposed to IPNV at 5, 7 and 10 days post-hatch. For each strain and age group, IPNV was added to three bottles to give 10^6 pfu/ml of water. A similar volume of PBS was added to three control bottles. After 6 hours, and daily thereafter for three weeks, half of the water in each bottle was replaced, debris was removed, and newly hatched brine shrimp were provided as forage for the striped bass fry. All dead fish were removed, stored at 4 or -20°C, and assayed for IPNV using

the simultaneous seeding procedure. The length of storage varied from 1 - 90 days.

Maryland strains of striped bass fry (Chesapeake and Delaware Canal [C & D] and Nanticoke [NAN] River) were divided into groups of 30 fry that were placed in 200 ml culture bottles containing Chesapeake Bay estuarine water (18 - 23°C). The C & D canal striped bass fry were challenged with IPNV at 1, 5, 15 and 20 days post-hatch. The NAN striped bass fry were exposed to IPNV at 10, 15 and 20 days post-hatch. The challenge protocol and daily care were as described above, with the exception that daily water changes replaced 75% (instead of 50%) of the water. Dead striped bass were stored for 0 - 3 days at 4°C prior to being assayed using the simultaneous seeding method. At the end of three weeks, 98% of the surviving striped bass were assayed for virus. Remaining survivors were assayed for virus and virus-neutralizing antibody six months after IPNV challenge.

To test the effects of an abrupt shift in pH on mortality in IPNV-exposed striped bass fry, five-day-old striped bass fry (C & D strain) were challenged with IPNV and maintained as described above. The only difference occurred on day five after viral exposure when 50% of the water (pH 7.1) was replaced with water to which sulfuric acid had been added to bring the pH of the water to pH 6.3. After 24 hours, the acidified water (now pH 6.5) was replaced with ambient water. Fish that died were collected

daily for three weeks and immediately assayed for virus using the simultaneous seeding method.

Waterborne IPNV Challenge of Striped Bass Fingerlings

Twenty-six-day-old striped bass were divided into 12 groups of 50 fish each. Six groups were kept at 12°C, and six were kept at 22°C. Water flow to all tanks was stopped for six hours, and the water aerated. At each temperature, two tanks were seeded with 10^4 pfu of IPNV per ml water, two tanks received phosphate buffered saline (sham controls), and two tanks of striped bass served as treatment controls. Dead fish were collected twice daily for three weeks and stored at -20°C until assayed for virus using the plaque assay. Samples were stored for 7 - 240 days.

Six-month-old striped bass fingerlings were assigned to three groups of 12 striped bass. One group of striped bass was exposed to a 6-hour static immersion in 10^6 pfu of IPNV per milliliter of water. Phosphate buffered saline was added to the 6-hour static bath of the sham control group. The treatment control group of striped bass underwent six hours of static bath. Tanks were checked daily for mortality. At four weeks post exposure, striped bass were assayed for IPNV and for virus-neutralizing antibody.

Virus Inoculation of Striped Bass Fingerlings

An alternative method of IPNV exposure was used for striped bass fingerlings two months and older. Rather than being immersed in IPNV, each fish received an intraperitoneal (i.p.) injection with IPNV. For all injection and sampling procedures, striped bass were anesthetized with tricaine methanesulfonate (MS-222).

Striped bass, 60, 90, 120, 150 and 180 days old, were placed into groups of 50 fish. At each age, one group of striped bass received an injection of 0.05 ml of PBS containing 0, 10^3 , 10^5 , or 10^6 pfu of IPNV. Treatment controls were anesthetized and returned to the tank. Dead fish were collected daily for four weeks, and stored at -20°C for 1 - 7 days until they were assayed for infectious virus. At monthly intervals, survivors were bled for virus-neutralizing antibody, and tissues were assayed for infectious virus.

To determine the effect of an abrupt temperature shift on mortality in IPNV-infected striped bass, 24 six-month-old striped bass were acclimated for two weeks to 12°C and an additional 24 fish were maintained at 22°C . All the fish were anesthetized and inoculated i.p. with 10^6 pfu of virus. Two weeks later, half of the fish held at half of the fish held at 12°C were transferred to 22°C , and half of the fish held at 22°C were transferred to 12°C . Fish were observed daily for mortality. After one month, survivors from each group were bled and assayed for virus.

Histological Examination

The histology of IPNV-infected striped bass was examined. Striped bass fingerlings were selected at monthly intervals after IPNV injection, incised along the ventral abdomen, and immersed in Bouin's fluid fixative (Luna, 1968). Fingerling tissues were embedded in paraffin. Striped bass fry (3 - 6 per day) were fixed in a solution of formalin and glutaraldehyde (4:1) and embedded in hydroxyethyl methacrylate. Blocks were sectioned at 4 - 6 microns, stained with hematoxylin, eosin and phloxine (Thompson, 1966) and examined by light microscopy.

Virus Transmission Studies

Oral Transmission of IPNV to Striped Bass

This study was conducted to ascertain if striped bass could become infected with IPNV by consuming IPNV-containing food. Six-month-old striped bass were tagged and placed into four tanks. Each tank contained six striped bass. Three-month-old brook trout, each harboring $10^2 - 10^4$ pfu of IPNV, were added to the tanks. Each striped bass was observed to consume one or two trout. Striped bass that did not eat brook trout were removed from the experiment. For six months, the striped bass were assayed periodically for the presence of IPNV and virus-neutralizing antibody.

Vertical IPNV Transmission in Striped Bass

A series of experiments was conducted to investigate if vertical transmission of IPNV occurred in striped bass. To determine if striped bass that survived a natural IPNV episode actually shed IPNV in sex products, the following study was conducted. A population of two-year-old striped bass from which IPNV was originally isolated (Schutz et al., 1984) was sampled. Milt was manually expressed from males; however, since striped bass females mature at the age of three plus years (Setzler et al., 1980), eggs were not available. Because preliminary results showed that IPNV can be recovered from striped bass kidney and, therefore, might be shed in the urine; urine was manually expressed and collected from sexually immature striped bass. Fourteen urine and 20 milt samples were tested for the presence of IPNV using the simultaneous seeding assay. Samples were processed within two hours of collection.

To determine if IPNV-infected striped bass transmit IPNV in their sex products, five-year-old striped bass were injected i.p. with 10^6 pfu of IPNV in December 1984, and spawned in April and May 1985. Samples of sex products, fertilized eggs and offspring were assayed for IPNV using the plaque method.

To investigate whether IPNV-infected striped bass sex products would result in IPNV-infection of the offspring, sex products were collected from spawning striped bass adults caught in the Nanticoke River (MD). Subsamples of

the eggs and milt were tested for the presence of IPNV. The remaining portions of eggs and milt were used to produce fertilized eggs. Eggs were dipped once in clean water and mixed with milt for fertilization. Additional water was added to the fertilized eggs and the mixture was placed in buckets and aerated. Striped bass fry hatched 2 days later. Treatment groups included (1) virus-exposed eggs plus virus-free milt--eggs were briefly mixed with virus (10^6 pfu/ml final IPNV concentration) before being dipped in water and then fertilized; (2) virus-free eggs plus virus-exposed milt--sperm was mixed with IPNV (10^6 pfu/ml final concentration of IPNV) and added to the eggs; and (3) treatment controls---virus-free eggs were mixed with virus-free milt. Periodically, fertilized eggs and fry were tested for the presence of IPNV using the simultaneous seeding assay.

Transmission of IPNV from Striped Bass to Brook Trout

This experiment was performed to ascertain whether IPNV could be transmitted from IPNV-infected striped bass to brook trout located downstream from the striped bass. Brook trout were utilized in this study because they are extremely susceptible to IPNV infection (Silim et al, 1982). Four-month-old striped bass were inoculated with 10^6 pfu of virus. At two months post inoculation, the internal organs from three IPNV-infected striped bass, and fecal samples from five fish were assayed for IPNV.

Fifteen IPNV-infected striped bass were placed in a tank. Water (12°C) from the tank containing striped bass flowed into a tank that contained 20 seven-month-old brook trout. Every two weeks, 3 - 4 trout were assayed for IPNV using the plaque assay.

Humoral Response of Striped Bass to IPNV

Early IPNV Titers and Neutralizing Antibody

The tissue levels of IPNV and circulating virus-neutralizing antibodies during the first ten days of IPNV infection were monitored in four-month-old striped bass fingerlings inoculated i.p. with 10^6 pfu of IPNV. For ten days, 3 - 4 fish daily were exsanguinated from the severed caudal peduncle and dissected. The kidneys, spleen, intestines, feces, and buffy coat were assayed for IPNV. Titers of virus-neutralizing antibody were measured in the blood samples from individual or pools of two fish.

Exogenous Steroids and Levels of Neutralizing Antibody in Striped Bass Challenged with IPNV

One investigation was conducted to determine the effect of exogenous corticosteroids on the development of viremia and virus-neutralizing antibodies in IPNV-inoculated striped bass. Striped bass yearlings were weighed, had a blood sample taken, and were divided into four groups of six fish each. The treatment groups were (1) sham control--fish were given two i.p. injections of PBS 24 hours apart; (2) steroid control--fish were

injected i.p. with the corticosteroid triamcinolone acetomide (100 mg/kg) followed 24 hours later with an i.p. injection of PBS; (3) virus control--fish were given a single i.p. injection with 10^7 pfu of IPNV; and (4) steroid + virus--fish were injected i.p. with steroid (100 mg/kg) 24 hours before i.p. inoculation with 10^7 pfu of IPNV. Half of the fish in each group were bled at 3 days post inoculation (dpi) and weekly thereafter for five weeks. Fish in the other half of each group were bled at 7 dpi and weekly thereafter, for five weeks. Blood plasma and leukocytes were assayed for IPNV. Levels of circulating of virus-neutralizing antibody were also measured.

Another study was conducted to determine if exogenous steroids affected levels of virus-neutralizing antibodies in IPNV-carrier striped bass. Striped bass fingerlings were inoculated i.p. with 10^5 pfu of IPNV. Eleven months later, the fish were weighed, bled, and injected i.p. with triamcinolone acetomide (100 mg/kg). Striped bass were bled twice weekly for three weeks. Titers of IPNV-neutralizing antibody were determined.

Antibody Response of Striped Bass to Second IPNV Challenge

The purpose of this study was to investigate the humoral response of IPNV-inoculated striped bass that were given a second exposure to IPNV, either by injection or by immersion challenge. For one part of this experiment, yearling striped bass were given an i.p. inoculation

containing 10^7 pfu of IPNV. Blood samples were taken twice weekly for five and one half weeks. The fish were allowed to rest for five weeks prior to the second i.p. injection with IPNV. Three months after the first virus injection, the striped bass received an i.p. inoculation of 10^6 pfu of IPNV. Blood samples were taken twice weekly for three weeks, and periodically for nine additional weeks. Levels of virus-neutralizing antibody were measured.

In a second part of the experiment, IPNV-inoculated striped bass were given a second IPNV challenge by the waterborne route. Five-month striped bass fingerlings were given i.p. inoculation with 10^5 pfu of IPNV. Fourteen months later, a blood sample was taken from these fish. The fish were immersed for 5 minutes in water containing 10^5 pfu of IPNV per ml. Blood samples were taken twice weekly for three weeks and assayed for levels of virus-neutralizing antibody.

Survey of Chesapeake Bay Striped Bass
for IPNV and Virus-Neutralizing Antibody

Sampling Young-of-Year Striped Bass

Young-of-year striped bass were caught using a 100 foot, 50 mm mesh seine at sites in traditionally important nursery areas in the Chesapeake Bay (MD). Striped bass were either placed immediately on ice, or a 0.04 ml blood sample was collected by venipuncture of the caudal vein. Fish that were bled were returned to the water. Striped

bass tissues were assayed for IPNV. Plasma samples were assayed for neutralizing activity against the striped bass isolate of IPNV (IPNV-Sb) and the european IPNV isolate (IPNV-Ab).

Sampling Yearling Striped Bass

Yearling striped bass from northern Chesapeake Bay were caught by hook and line. A 0.04 ml blood sample was obtained by venipuncture of the caudal vein, and the fish were returned to the water. Plasma samples were tested for virus neutralizing activity against both IPNV-Sb and IPNV-Ab.

Sampling Adult Striped Bass

Adult striped bass were caught in gill nets located in the Chesapeake Bay. Kidneys, spleen, gonads, and intestines were excised, placed in sterile plastic bags, and stored at 4°C for 1 - 3 days prior to assay for IPNV. Blood samples were collected from the caudal vein in sterile glass tubes and the serum tested for virus-neutralizing activity against both IPNV-Sb and IPNV-Ab.

Procedures that Affect IPNV Recovery

Tissue Site of IPNV in Striped Bass

When monitoring fish populations for IPNV, tissue samples should be taken from which virus can be recovered most frequently. An investigation was conducted to

ascertain which striped bass tissues harbor IPNV.

Individual organs, fat, feces, and blood, were removed from IPNV-infected striped bass, and assayed for infectious virus.

Storage Conditions of IPNV-infected Homogenates

The lability of the striped bass isolate of IPNV (IPNV-Sb) in homogenates of IPNV-infected striped bass was studied. Pools of internal organs from IPNV-inoculated striped bass were homogenized and clarified as previously described. Aliquots of the supernatant fluid were placed in sterile glass vials and stored at 4, -20 or -70°C.

An experiment was conducted to investigate whether the type of container in which the homogenate of the internal organs from IPNV-infected striped bass was stored altered the amount of IPNV detected. The tissue homogenate from individual IPNV-carrier striped bass was divided into 7 aliquots. One aliquot was assayed immediately for IPNV using the plaque method. Three aliquots were stored in plastic bags and three were stored in glass vials. Two aliquots (one in glass vial, one in plastic) from each fish homogenate were stored at each of three temperatures (4, -20 and -70°C) prior to virus assay.

Storage Temperature of Whole IPNV-Infected Striped Bass

Sampling fish for IPNV frequently involves taking whole fish or tissue samples in the field and storing the samples until they can be assayed for virus. An

investigation was conducted to determine the effect of different storage temperatures on recovery of infective IPNV from virus-infected striped bass. For this experiment, IPNV-infected striped bass were placed in individual plastic bags and stored at either 4, -20 or -70°C for two to fourteen days. After storage, frozen fish were allowed to soften at 4°C, and then the internal organs from all fish were excised, and assayed for virus infectivity.

Detection of IPNV-Carriers after Steroid Injection

A study was conducted to investigate whether exogenous corticosteroids would enhance recovery of IPNV from chronic IPNV-infected striped bass. Fifteen months after IPNV-inoculation, three striped bass were placed in each of five tanks. All fish were weighed and injected i.p. with triamcinolone acetomide (10 mg/kg). One group was immediately exsanguinated and assayed for IPNV. The other groups were assayed for IPNV and virus-neutralizing antibody at 3, 7, 14 and 21 days following steroid injection.

CHAPTER THREE RESULTS

Virus Infection Studies of Striped Bass

A series of IPNV challenge trials was conducted to determine if IPNV induces increased mortality in virus-exposed striped bass. When 1- to 20-day-old striped bass from four different strains were immersed in IPNV the resulting mortality was not significantly different from that of the unchallenged controls ($p < 0.01$, analysis of variance [ANOVA]). Even when five-day-old IPNV-exposed fry were subjected to an abrupt drop in pH (0.8 units), no statistical difference was observed in the mortality of control and IPNV-challenged fry. Mortality in different trials was unpredictable, but within a trial the pattern of mortality of virus-challenged and control fish were not significantly different (Figure 1). Virus was recovered from virus-exposed fish that died but was never isolated from control fish. When survivors were assayed for virus three weeks post-challenge, IPNV was recovered only from fry that had been challenged at one day post-hatch (data not shown). Virus was not recovered any of the surviving fish six months after waterborne challenge.

Twenty-six-day-old striped bass exposed to IPNV by immersion and held at 12 or 22°C exhibited no difference in mortality compared to control groups ($p < 0.01$) (Figure 2).

Figure 1: Percent daily mortality of striped bass fry during the 21 days following exposure to 10^5 plaque forming units of infectious pancreatic necrosis virus (IPNV) per milliliter of water ($\square-\square$) or to phosphate buffered saline ($+---+$). Sixty or 180 striped bass were exposed to virus in each trial. There was no significant difference between the mortality in IPNV-exposed and unchallenged striped bass ($p < 0.01$; tested by analysis of variance). Results from representative trials are presented.

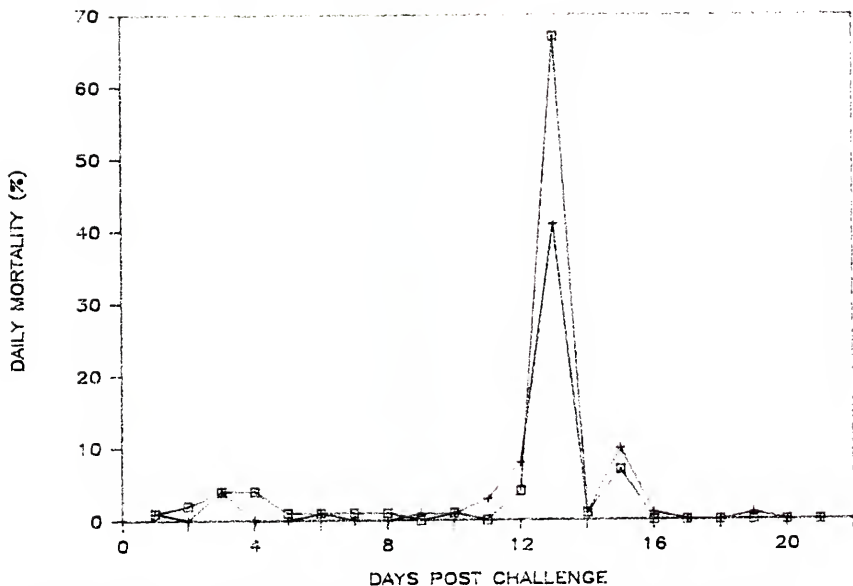


Figure 1 A. Chesapeake and Delaware Canal (MD) striped bass fry were exposed to IPNV at one day post-hatch.

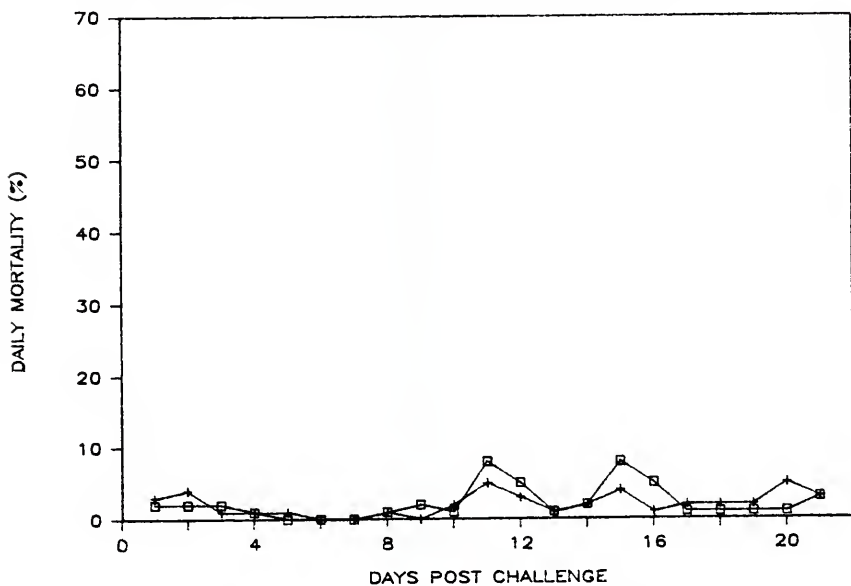


Figure 1 B. Chesapeake and Delaware Canal striped bass fry were exposed to IPNV at five days post-hatch.

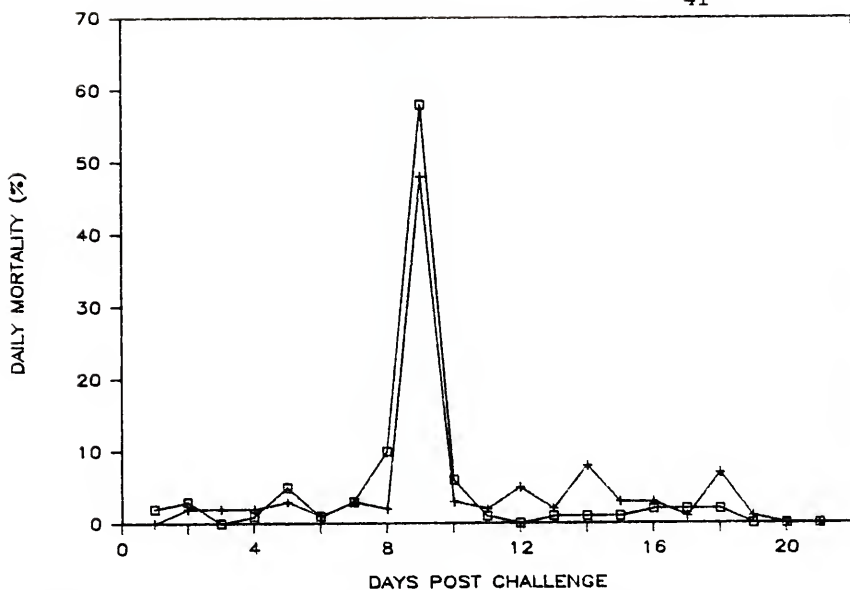


Figure 1 C. Florida striped bass fry were exposed to IPNV at seven days post-hatch.

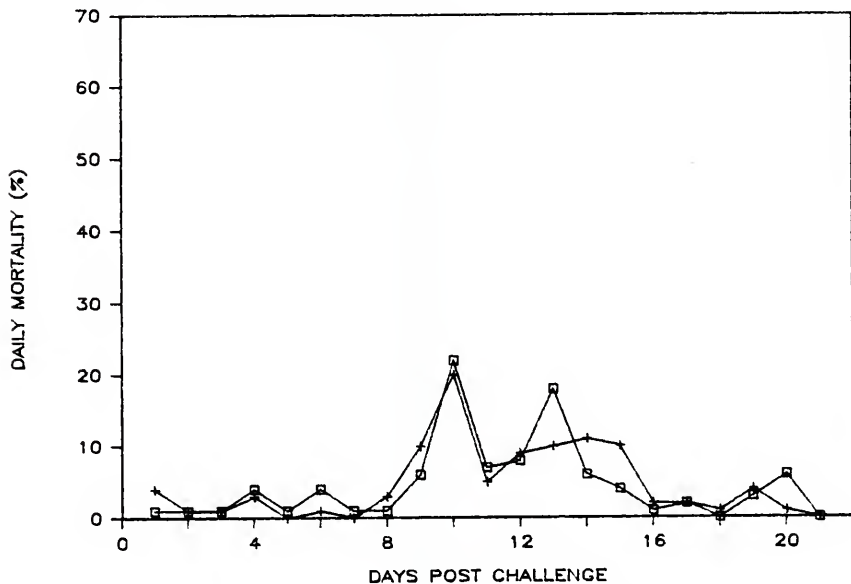


Figure 1 D. Georgia striped bass fry were exposed to IPNV at ten days post-hatch.

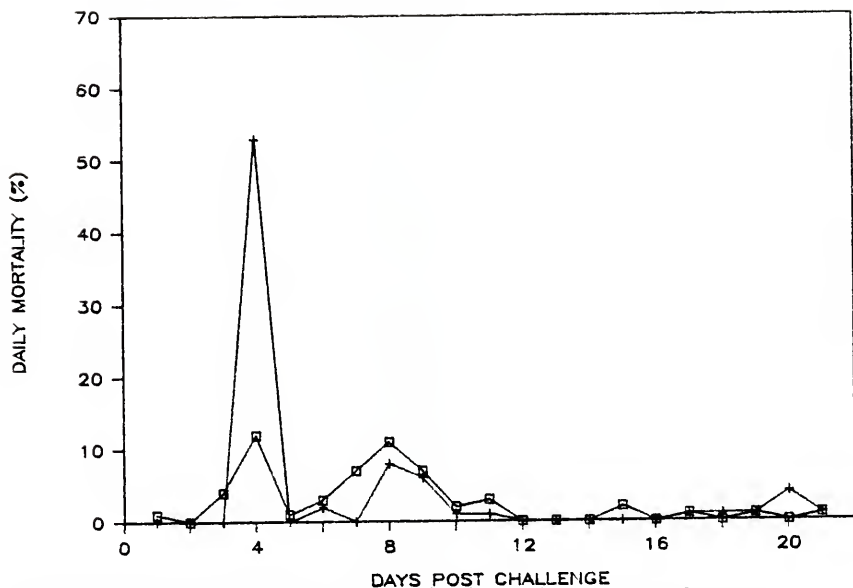


Figure 1 E. Nanticoke River (MD) striped bass fry were exposed to IPNV at 15 days post-hatch.

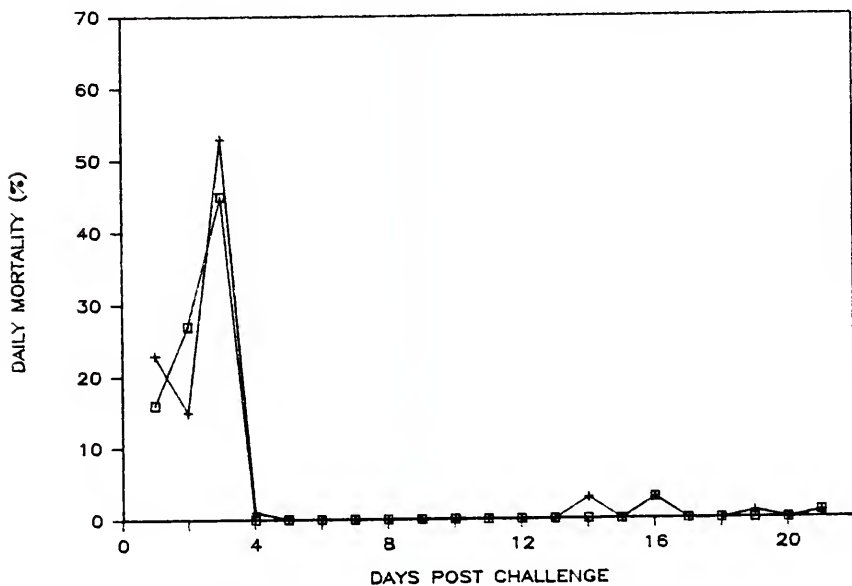


Figure 1 F. Nanticoke River striped bass fry were exposed to IPNV at 20 days post-hatch.

Figure 2: Percent daily mortality of striped bass fingerlings exposed at 26 days post-hatch to 10^4 plaque forming units of infectious pancreatic necrosis virus per milliliter of water ($\square-\square$). Sham controls ($+ \rightarrow +$) were exposed to virus-free phosphate buffered saline (PBS). Treatment controls ($x \rightarrow x$) were held for four hours in a static, aerated bath without PBS or virus. Each experimental group contained 100 striped bass. There was no significant difference between mortality of virus-exposed and nonexposed controls ($p < 0.01$; tested by analysis of variance) at either temperature.

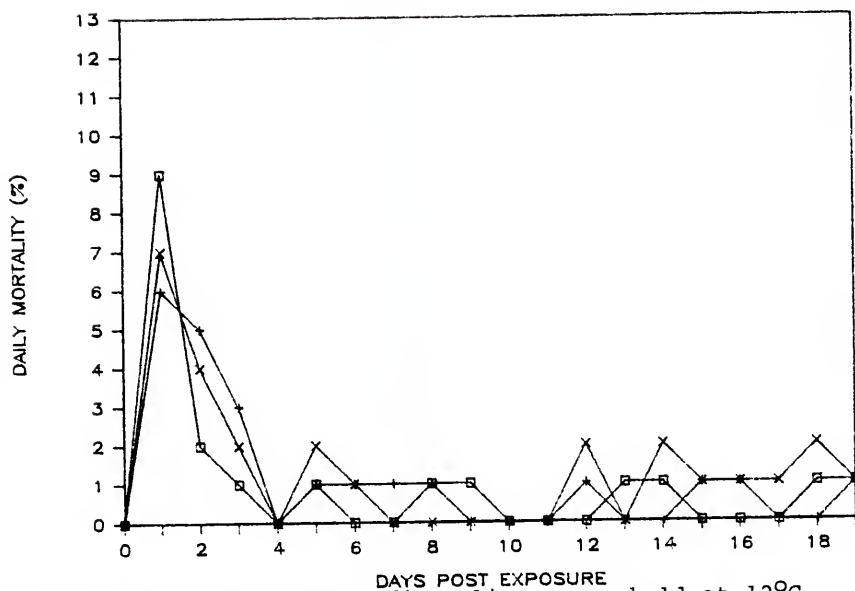


Figure 2 A. Striped bass fingerlings were held at 12°C.

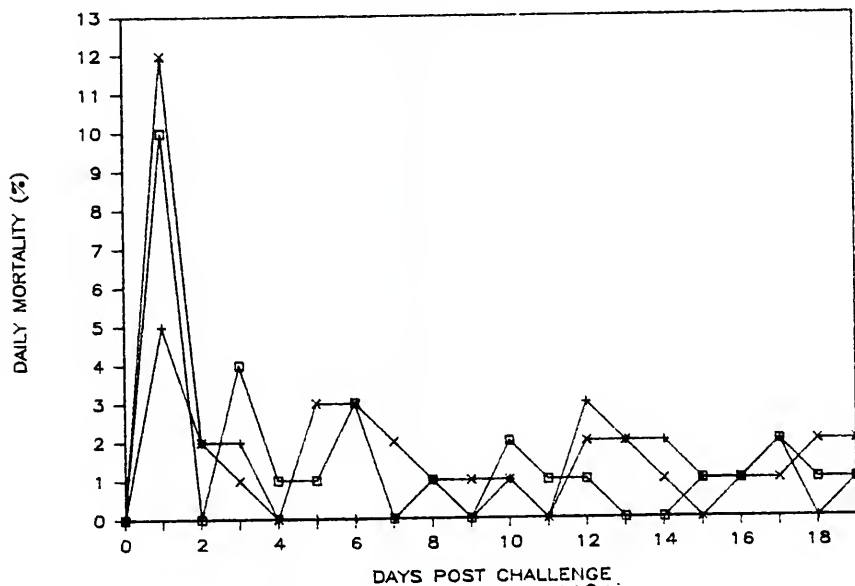


Figure 2 B. Fingerlings were held at 22°C.

In addition, no virus was recovered from any fish that died. No deaths occurred in six-month-old striped bass that were challenged with IPNV by immersion and no virus was recovered from any of these fish.

Mortality did not increase in striped bass fingerlings that were given IPNV by intraperitoneal (i.p.) inoculation at either 60, 120, 150 and 180 days post-hatch (Table 1). Even among IPNV-injected striped bass that underwent an abrupt 10°C change in water temperature, mortality was not significantly different from that of controls. Virus was recovered from IPNV-injected fish that died but was not isolated from any controls (Table 2). At one month after IPNV injection, virus titers of survivors were similar to those of IPNV-inoculated striped bass that died during the first month after injection (Table 3). Even levels of IPNV in virus-inoculated striped bass subjected to changes in water temperature were not significantly different ($p < 0.01$, ANOVA) (Table 4). Virus was isolated from surviving IPNV-inoculated striped bass for 14 months post-inoculation (Table 3). Circulating IPNV-neutralizing antibody was found in more than 75% of the IPNV-carrier striped bass tested during the 14 months after IPNV exposure. No IPNV-induced histological lesions were observed in any sections examined from IPNV-exposed striped bass, regardless of age or route of exposure.

Table 1: Percent cumulative mortality in striped bass fingerlings following intraperitoneal injection of infectious pancreatic necrosis virus (IPNV).

AGE ^a	CONTROL		VIRUS INOCULUM (pfu)		
	TREAT- MENT	SHAM	10 ³	10 ⁵	10 ⁶
60	ND ^b	48 ^c	38	40	ND
90	28	26	24	26	14
120	20	18	2	16	14
150	0	0	0	2	0
180	8	0	0	0	0

^aAt the indicated days post-hatch, groups of 50 striped bass were anesthetized and given intraperitoneal (i.p.) injections containing the indicated plaque forming units (pfu) of IPNV. Treatment controls were anesthetized only. Sham controls were injected with phosphate buffered saline (virus diluent). Fish were maintained at 22°C.

^bNot done.

^cPercentage of striped bass that died in the 28 days following inoculation.

Table 2: Range of virus titers detected from striped bass fingerlings that died following intraperitoneal injection of infectious pancreatic necrosis virus.

AGE ^a	CONTROL		VIRUS INOCULUM (PFU)		
	TREAT- MENT	SHAM	10 ³	10 ⁵	10 ⁶
60	ND ^b	NV ^c	10 ² ^d	10 ³ -10 ⁵	ND
90	NV	NV	NV	NV	10 ² -10 ⁴
120	NV	NV	NV	10 ³ -10 ⁶	10 ⁵ -10 ⁷
150	- ^e	-	-	10 ⁵	-
180	NV	-	-	-	-

^aAt the indicated days post-hatch, striped bass were given intraperitoneal (i.p.) injections of the indicated plaque forming units (pfu) of infectious pancreatic necrosis virus (IPNV). Treatment controls were anesthetized only. Sham controls were injected i.p. with phosphate buffered saline (virus diluent). Fish were maintained at 22°C. Dead fish were assayed for virus using the plaque assay method that detected titers greater than or equal to 5×10^1 pfu/g.

^bNot done.

^cNo IPNV was recovered from striped bass that died during the first 28 days following injection.

^dMagnitude of IPNV titers (pfu/g of tissue) that were recovered from striped bass that died during the first 28 days following injection with IPNV.

^eNo fish died in this group.

Table 3: Range of virus titers in striped bass fingerlings surviving intraperitoneal injection of infectious pancreatic necrosis virus.

MONTHS ^b	VIRUS INOCULUM (PFU) ^a		
	10 ³	10 ⁵	10 ⁶
1	NV ^c	10 ³ -10 ⁴ ^d	10 ³ -10 ⁶
2	10 ²	10 ² -10 ⁴	10 ³ -10 ⁶
3	10 ³	10 ⁴	10 ³ -10 ⁴
4	NV	10 ³ -10 ⁴	10 ³ -10 ⁴
6	10 ¹ -10 ²	NV-10 ²	NV-10 ³
12	NV-10 ¹	NV-10 ²	NV-10 ¹
14	ND ^e	ND	NV-10 ²

^aStriped bass fingerlings received an intraperitoneal inoculation with the indicated plaque forming units (pfu) of infectious pancreatic necrosis virus (IPNV). Fish were maintained at 22°C.

^bMonths following intraperitoneal injection that surviving striped bass were assayed for IPNV using the plaque method that detected titers equal to or greater than 5×10^1 pfu/g.

^cNo IPNV was recovered from surviving fingerlings.

^dRange in IPNV titers (pfu/gram of tissue) in striped bass fingerling survivors that were assayed for virus.

^eNot done.

Table 4: Virus titers of individual striped bass that were given an intraperitoneal injection of infectious pancreatic necrosis and subjected to a change in water temperature.

TEMPERATURE ^a			
22	12	22 --> 12	12 --> 22
2.7×10^4	9.7×10^4	2.3×10^5	1.2×10^5
2.9×10^4	2.9×10^4	4.9×10^3	2.6×10^4
3.9×10^4	4.5×10^4	1.1×10^6	3.3×10^4
3.0×10^4	1.4×10^4	2.2×10^5	NV ^b
NV	3.2×10^4	1.9×10^4	8.2×10^3
7.2×10^4	2.0×10^6	1.9×10^4	9.2×10^4
NV	5.0×10^6	2.8×10^4	8.0×10^3
1.2×10^5	6.4×10^5	4.8×10^3	2.1×10^4
-----	-----	-----	-----
4.0×10^4 ^c	9.8×10^5	2.0×10^5	3.9×10^4
4.0 ^d	1.7	3.6	4.4

^aSix-month-old striped bass were given an intraperitoneal injection of 10^6 plaque forming units (pfu) of infectious pancreatic necrosis virus (IPNV). Fish were held at either 12°C or 22°C. Two weeks after the IPNV inoculation, some fish were transferred into water of a higher or lower temperature. Two weeks after the temperature change, fish were assayed for IPNV using the plaque method that detected titers equal to or greater than 5×10^1 pfu/g. Virus titers were expressed as pfu per gram of tissue.

^bNo virus was detected.

^cMean IPNV titer for each group. Group means were not significantly different ($p < 0.01$) as determined by analysis of variance.

^dStandard deviation of mean IPNV titer.

Transmission Studies of IPNV in Striped Bass

Oral Transmission of IPNV to Striped Bass

To demonstrate that IPNV can be transmitted to striped bass by contaminated forage, six-month-old striped bass were allowed to consume brook trout carrying between 10^2 - 10^4 pfu of IPNV. Virus was recovered from apparently healthy striped bass eight months after exposure (Table 5). Virus-neutralizing antibody was detected in all striped bass that consumed IPNV-infected brook trout.

Vertical Transmission of IPNV in Striped Bass

To determine if striped bass survivors from natural IPNV infection shed virus in their urine or milt, samples were taken from the population of two-year-old striped bass from which IPNV had originally been isolated (Schutz et al., 1984). No IPNV was detected in any of the urine and milt samples.

Experiments were conducted to investigate whether IPNV-infected striped bass adults would transmit IPNV via their sex products to their offspring. The eggs, milt, fertilized eggs and offspring from striped bass adults that had received i.p. inoculation with IPNV were tested for virus. Virus (10^1 - 10^3 pfu/gram of tissue) was recovered from the internal organs of the adults, but no IPNV was detected in any other samples (Table 6). When IPNV was added to eggs or milt, virus was not recovered from the resultant offspring. Virus was only recovered from

Table 5: Range in virus titer in striped bass following ingestion of brook trout that contained infectious pancreatic necrosis virus.

WEEKS POST ^a EXPOSURE	VIRUS TITER ^b	NUMBER TESTED
1	10^1	1
2	10^1-10^3	3
3	10^1-10^3	2
4	10^1-10^3	2
12	10^1-10^2	2
33	10^1	1

^aSix-month-old striped bass were fed brook trout, each of which contained $10^2 - 10^4$ plaque forming units (pfu) of infectious pancreatic necrosis virus (IPNV). At the indicated weeks after virus ingestion, striped bass were assayed for IPNV by the plaque method.

^bMagnitude of titer expressed as pfu of IPNV per gram of striped bass tissue.

Table 6: Recovery of infectious pancreatic necrosis virus (IPNV) from samples taken during investigations of vertical transmission of IPNV in striped bass.

SAMPLES	VIRUS RECOVERED
Adults inoculated with IPNV ^a	
Internal Organs	Yes
Sex Products (Eggs and Milt)	No
Fertilized Eggs	No
Fry	No
Noninoculated Adults ^b	
Sex Products	No
IPNV added to Eggs ^c	
Fertilized Eggs	Yes
Fry	No
IPNV added to Milt ^d	
Fertilized Eggs	No
Fry	No

^aFive-year-old, hatchery-reared striped bass were given an intraperitoneal injection with 10^6 plaque forming units (pfu) of IPNV. Fish were spawned six months later. Samples were assayed for virus using the plaque method.

^bSex products (eggs and milt) were obtained from spawning striped bass caught in the Chesapeake Bay (MD). Homogenates of eggs, milt, fertilized eggs, and larvae were assayed for IPNV using the simultaneous seeding method. Samples were considered positive for IPNV if cytopathic effects (CPE) was observed during two blind passages. If no CPE developed, the sample was recorded to be negative.

^cEggs were exposed to 10^5 pfu of IPNV/ml immediately prior to mixing with virus-free milt.

^dMilt was exposed to 10^5 pfu of IPNV/ml immediately prior to mixing with virus-free eggs.

fertilized eggs when virus-exposed eggs were fertilized with virus-free milt. None of the offspring started to feed and all died within three weeks.

Transmission of IPNV from Striped Bass to Brook Trout

To determine whether IPNV-infected striped bass shed sufficient virus to infect susceptible fish located downstream, brook trout were placed below IPNV-infected striped bass whose feces contained 10^4 - 10^5 pfu/g. One of four trout tested after six weeks had 10^2 pfu of IPNV/g of pooled internal organs. Virus was not recovered from trout tested at two, four and eight weeks of IPNV-exposure.

Humoral Response of Striped Bass to IPNV

Early Humoral Response to IPNV Challenge

To monitor early levels of IPNV and circulating virus-neutralizing antibodies in striped bass, four-month-old striped bass were inoculated i.p. with 10^6 pfu of IPNV and 3 - 4 fish were assayed each day for 10 days. Titers of virus remained relatively constant during the first ten days (Table 7) and were of the same magnitude as IPNV titers in IPNV-inoculated striped bass tested two months after injection (Table 3). Virus-neutralizing antibody was first detected seven days post inoculation (dpi) (Table 7).

Table 7: Detection of infectious pancreatic necrosis virus (IPNV) and virus-neutralizing antibody in IPNV-injected striped bass fingerlings.

DPI ^a	IPNV ^b	TITER ^c	ANTIBODY ^d	TITER ^e
1	3 / 3	10 ⁵ -10 ⁶	NT ^f	-
2	3 / 3	10 ⁵	0 / 3	-
3	4 / 4	10 ⁵ -10 ⁶	0 / 2	-
4	4 / 4	10 ³ -10 ⁶	0 / 3	-
5	4 / 4	10 ⁵	0 / 2	-
6	3 / 3	10 ⁵ -10 ⁶	0 / 3	-
7	4 / 4	10 ⁵ -10 ⁶	1 / 3	500
8	4 / 4	10 ⁴ -10 ⁵	2 / 3	200-700
9	4 / 4	10 ⁴ -10 ⁵	2 / 3	500
10	4 / 4	10 ⁵	2 / 4	750-1000

^aDays post injection (intraperitoneal) of 10⁶ plaque forming units (pfu) of IPNV.

^bNumber of four-month-old striped bass that had IPNV in their tissues per number of fish assayed for virus using the plaque method.

^cRange in IPNV titer (pfu per gram of tissue).

^dNumber of blood samples (diluted 1:100) that neutralized more than 50% of total IPNV plaques per number of blood samples tested.

^eRange in titer of IPNV-neutralizing antibody.

^fNot tested.

Effect of Steroids on Titers of Circulating Virus and
IPNV-Neutralizing Antibody

The effect of exogenous steroid on viremia and on the development of virus-neutralizing antibody was investigated using yearling striped bass that received an i.p. injection of steroid 24 hours prior to receiving an i.p. injection with IPNV. Blood samples were taken weekly from individual fish. Viremia was detected for two weeks in IPNV-inoculated striped bass that had received steroid (Table 8), but for only one week in IPNV-inoculated striped bass that did not receive steroid. Virus was recovered more frequently from the buffy coat (leukocytes) than from the plasma (Table 8). Circulating IPNV-neutralizing antibody was first detected at 10 dpi in IPNV-inoculated fish that received steroid (Figure 3) compared to 7 dpi in IPNV-inoculated fish not treated with steroid (Figure 3). Levels of virus-neutralizing antibody in the IPNV-injected striped bass treated with steroid were generally lower than those in virus-injected fish that did not receive steroid. Also, antibody titers peaked later (about 4 weeks post inoculation) in steroid treated fish compared to a peak at about 3 weeks in virus-injected striped bass that did not receive steroids. A noticeable, but not statistically significant, difference in antibody titers was observed between the two groups IPNV-injected striped bass that did not receive steroid. Striped bass that were bled at three dpi and weekly thereafter (Figure 3a) had somewhat higher

Table 8: Recovery of infectious pancreatic necrosis virus (IPNV) from the plasma and buffy coat of virus inoculated striped bass fingerlings.

TREAT- MENT	SAMPLE	DAYS POST INJECTION ^a					
		3	7	10	14	17	21
Steroid + IPNV ^b	buffy coat	3/3 ^c	3/3	0/3	1/3	0/3	0/3
	plasma	1/3	0/3	0/3	0/3	0/3	0/3
IPNV ^d	buffy coat	1/3	3/3	0/3	0/3	0/3	0/3
	plasma	0/3	0/3	0/3	0/3	0/3	0/3
Controls ^e	buffy coat	0/3	0/3	0/3	0/3	0/3	0/3
	plasma	0/3	0/3	0/3	0/3	0/3	0/3

^aStriped bass fingerlings received 10^7 plaque forming units (pfu) of IPNV or phosphate buffered saline by intraperitoneal (i.p.) inoculation. Blood samples, taken at the indicated days after IPNV injection, were assayed for IPNV using the plaque assay.

^b Striped bass fingerlings that received an i.p. injection with triamcinolone acetomide (100 mg/kg) 24 hours before i.p. inoculation with IPNV.

^cNumber of fish that were positive for IPNV per number of fish tested using the plaque assay.

^dStriped bass fingerlings that received only IPNV by i.p. injection.

^eStriped bass fingerlings that did not receive an injection of IPNV, but were injected i.p. with either PBS or steroid and PBS.

Figure 3: Titers of virus-neutralizing antibody in striped bass fingerlings that received an intra-peritoneal inoculation with 10^7 plaque forming units (pfu) of infectious pancreatic necrosis virus (IPNV). Fingerlings were injected with phosphate buffered saline (PBS) (\bigcirc) or with triamcinolone acetomide (100 mg/kg) (\square) 24 hours prior to viral inoculation. Antibody titers are expressed as 10^{-2} the serum dilution that neutralized 50% of total IPNV (about 80 plaques per well). The mean antibody titer for striped bass that received PBS is indicated by + and by — for fish that received steroid.

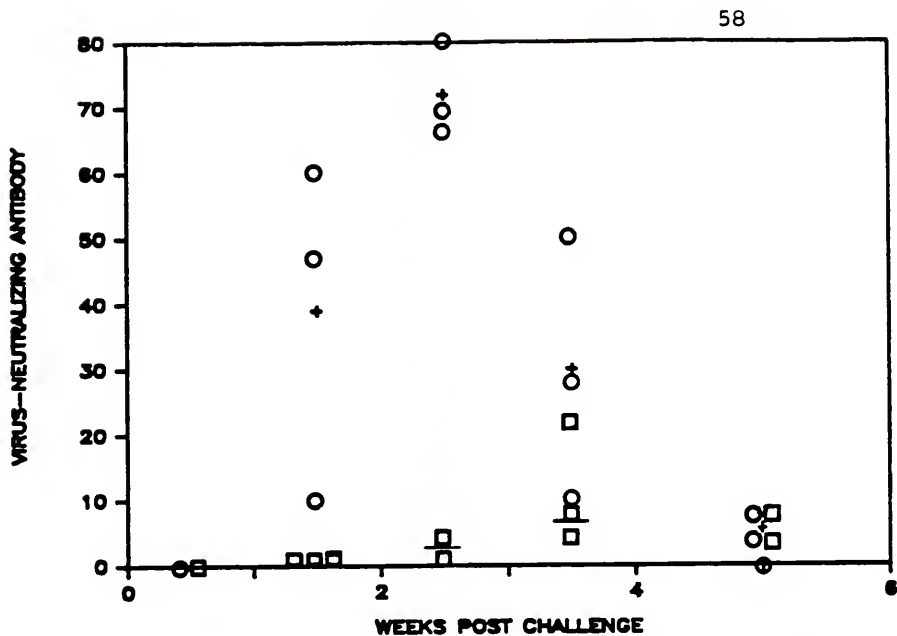


Figure 3 A. Fish that were sampled at weekly intervals beginning three days after IPNV inoculation.

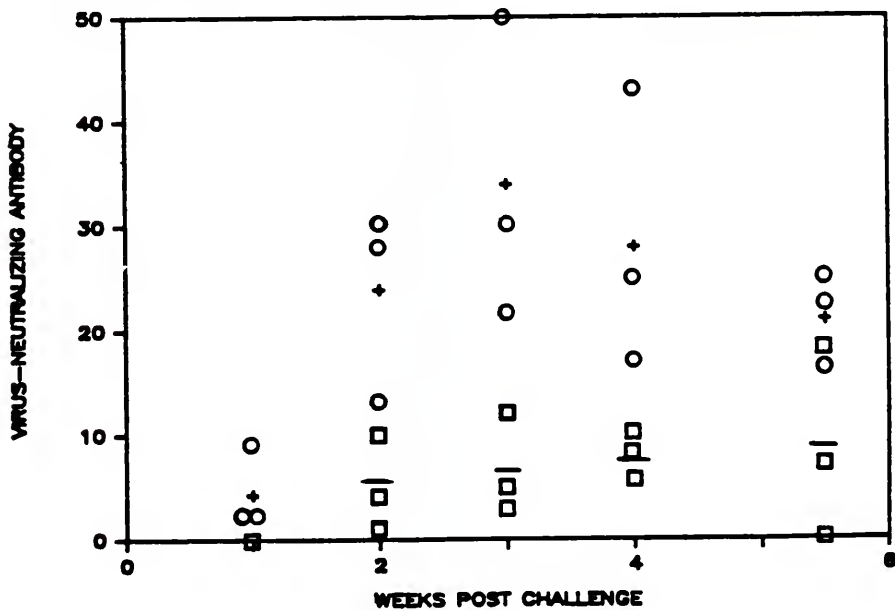


Figure 3 B. Striped bass that were sampled at weekly intervals after IPNV injection.

antibody titers than fish sampled at seven dpi and then weekly (Figure 3b).

Steroid treatment of chronic IPNV-carrier striped bass did not cause any change in levels of IPNV-neutralizing antibodies in these fish. Antibody titers remained between 100 and 500.

Administration of 100 mg/kg of triamcinolone acetomide resulted in a 96% loss among all steroid-injected striped bass over a three month period. The spleen and anterior kidneys of these fish were extremely hypocellular.

Antibody Response of Striped Bass Following a Second IPNV Challenge

To determine the humoral response of striped bass to a second IPNV exposure, two sets of experiments were performed. In one, IPNV-injected striped bass were given a second viral challenge by immersion. In the other, IPNV-injected striped bass received a second i.p. injection of IPNV. When IPNV-inoculated striped bass were given a waterborne IPNV challenge, antibody levels (100 - 800) remained unchanged after the second viral exposure.

In contrast, in IPNV-carrier fish that received a second injection with IPNV, antibody titers increased (Figure 4). Antibody levels in striped bass began to rise at seven dpi after the second IPNV inoculation and were considerably higher than levels detected after the first IPNV injection. After the second injection, antibody titers rose and fell twice over a nine week period.

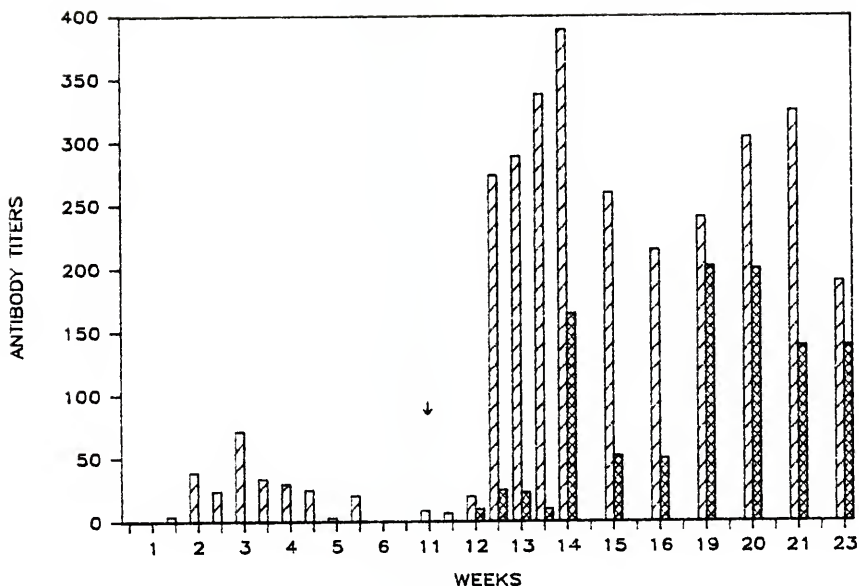


Figure 4: Virus-neutralizing antibody titers in striped bass fingerlings injected with infectious pancreatic necrosis virus (IPNV). Striped bass received 10^6 plaque forming units (pfu) of IPNV by intraperitoneal (i.p.) inoculation on day 0 (▨). Control fish received an i.p. injection with phosphate buffered saline (▩) on day 0. A second IPNV challenge (↓) was given at 11 weeks after the first injection. Control striped bass were injected i.p. with IPNV at week 11. Virus-neutralizing antibody titers are expressed as 10^{-2} the dilution of fish serum that neutralized 50% of the total viral plaques (about 80 plaques per well). Each bar represents the mean ($n = 1$ to 6) virus-neutralizing antibody titer of fish.

Survey of Chesapeake Bay Striped Bass

To determine whether wild striped bass have been exposed to IPNV, Chesapeake Bay (MD) striped bass of various ages were sampled. The tissues from some were assayed for virus. Blood samples were assayed for the presence of antibodies that would neutralize the striped bass isolate of IPNV (IPNV-Sb) but not the European Ab serotype. Virus was not recovered from any wild striped bass tested (Table 9). Specific IPNV-Sb neutralizing antibody was detected in 1- to 3-year-old striped bass caught during the winter of 1984 and in one young-of-year striped bass caught in 1985 (Table 10).

Procedures that Affect IPNV Recovery from Striped Bass Tissue Site of IPNV in Striped Bass

Tissues from IPNV-infected striped bass were assayed individually to determine those from which IPNV could be recovered most frequently. Virus was recovered from the anterior kidneys of all striped bass that were positive for IPNV but was never isolated from brain (Table 11). Virus was also recovered from other tissues but none with the consistency found for the anterior kidney (Table 11). Tissue virus titers ranged in magnitude from undetectable ($< 5 \times 10^1$ pfu/g) to 10^6 pfu/g.

Virus Recovery from Steroid Injected Chronic Carriers

This study was conducted to determine if exogenous corticosteroids would increase the percentage of virus

Table 9: Attempts to isolate infectious pancreatic necrosis virus (IPNV) from striped bass caught in the Chesapeake Bay (MD).

SURVEY DATE	LOCATION	YEAR-CLASS	VIRUS/SAMPLES ^a
Aug. 1984	Upper Bay	1984	0 / 39
Sept. 1984	Upper Bay	1984	0 / 66
Dec. 1984	Choptank River	1982 - 83	0 / 15
Feb. 1985	Upper Bay	1982 - 83	0 / 30

^aNumber of striped bass positive for IPNV per number of individual fish tested. Individual whole fish, or samples of kidney, spleen and feces were assayed for IPNV by the plaque method.

Table 10: Detection of neutralizing antibody specific for the striped bass isolate of infectious pancreatic necrosis virus (IPNV-Sb) in Chesapeake Bay striped bass.

SURVEY DATE	LOCATION	YEAR-CLASS	SAMPLES ^a
Dec. 1984	Choptank River	1982 - 83	9 / 49 (18%)
Feb. 1985	Upper Bay	1982 - 83	6 / 94 (6%)
July 1985	Upper Bay	1985	0 / 5
Aug. 1985	Choptank River	1985	1 / 45 (2%)
Aug. 1985	Upper Bay	1985	0 / 3
Sept. 1985	Upper Bay	1985	0 / 6
Sept. 1985	Upper Bay	1984	0 / 20

^aNumber of blood samples positive for IPNV-Sb neutralizing antibody per number of samples tested. Serum samples, diluted 1:100, were tested by the plaque method for neutralizing activity against IPNV-Sb and against the European isolate (IPNV-Ab). Samples were considered to be positive for specific IPNV-Sb neutralizing antibody if they neutralized more than 50% of IPNV-Sb, but did not neutralize IPNV-Ab. Total virus contained about 80 plaques per well.

Table 11: Striped bass tissues from which infectious pancreatic necrosis virus was isolated.

TISSUE ^a	# POSITIVE/	# TESTED ^b
Anterior kidney	29 / 29	(100%)
Spleen	20 / 25	(80%)
Blood	4 / 8	(50%)
Fat	2 / 4	(50%)
Liver	9 / 20	(45%)
Intestine	2 / 9	(22%)
Posterior kidney	4 / 20	(20%)
Heart	2 / 11	(8%)
Brain	0 / 13	(0%)

^aTissues from infectious pancreatic necrosis virus (IPNV) infected striped bass were assayed individually for virus by the plaque method.

^bNumber of tissues positive for IPNV per number assayed for IPNV.

isolation from striped bass that had been injected with IPNV 15 months earlier. Injection with triamcinolone acetamide (10 mg/kg) into these striped bass increased the percentage of virus-positive fish detected over time (Table 12); the peak occurred two weeks after steroid administration.

Virus Recovery from Stored IPNV-carrier Tissue Homogenates

Aliquots of tissue homogenates from individual IPNV-infected striped bass were stored under different conditions to determine if the stability of IPNV infectivity was affected. Virus infectivity was reduced in homogenates stored at 4°C (Table 13) but IPNV infectivity was not significantly different ($p < 0.01$, ANOVA) in homogenate samples stored at either -20°C or -70°C. The type of container (glass vial or plastic bag) did not result in a significant difference in virus infectivity ($p < 0.01$, ANOVA) (Table 14), although virus titers from homogenates stored at 4°C were significantly different ($p < 0.01$, ANOVA) from titers from homogenates stored at -20 or -70°C.

Recovery of IPNV from Stored Whole Striped Bass

In an additional study designed to determine if storage conditions affect the recovery of infectious IPNV from virus-infected striped bass, whole fish were stored for 2 to 14 days at 4, -20, and -70°C. Table 15 shows the virus titers of IPNV-infected striped bass fingerlings determined after storage. Virus titers were best maintained in IPNV-

Table 12: Recovery of infectious pancreatic necrosis virus (IPNV) from chronic virus-carrier striped bass following injection with steroid.

WEEKS ^a	# POSITIVE/# TESTED ^b	TITER ^c
0	0 / 4	NV ^d
0.5	0 / 3	NV
1	1 / 4	10 ²
2	3 / 4	10 ² - 10 ³
3	1 / 4	10 ²

^aNumber of weeks following intraperitoneal (i.p.) injection of triamcinolone acetomide (10 mg/kg) into striped bass that been injected i.p. with IPNV 14 - 15 months previously.

^bNumber of striped bass from which IPNV was recovered per number of striped bass assayed for IPNV by the plaque method.

^cRange in magnitude of virus titers expressed as plaque forming units of IPNV per gram of tissue (anterior kidney).

^dNo virus detected.

Table 13: Titers of virus of infectious pancreatic necrosis virus-infected striped bass tissue homogenates that were stored at different temperatures.

STORAGE ^a		FISH NUMBER				
		1	2	3	4	5
<u>0 days</u>						
		5×10^2 ^b	5×10^3	3×10^4	2×10^4	1×10^4
<u>2 days</u>						
4°C	NV ^c		1×10^3	6×10^3	4×10^3	4×10^3
-20°C	NV		7×10^3	3×10^4	5×10^4	7×10^4
-70°C	NV		8×10^3	5×10^4	5×10^4	3×10^4
<u>2 weeks</u>						
4°C	NV		NV	2×10^4	NV	1×10^4
-20°C		1×10^2	5×10^3	6×10^4	6×10^4	3×10^4
-70°C	NV		NV	5×10^4	2×10^4	1×10^3

^aLength of time and temperature at which aliquots of tissue homogenates from striped bass infected with infectious pancreatic necrosis virus (IPNV) were stored prior to assay for virus by the plaque method.

^bPlaque forming units of IPNV per gram of tissue (pooled internal organs) recovered from homogenates.

^cNo virus was recovered.

Table 14: Recovery of infectious pancreatic necrosis virus (IPNV) from striped bass tissue homogenates stored for 48 hours in different types of containers.

Sample	^a 4°C		-20°C		-70°C	
	V ^b	P ^c	V	P	V	P
1	NV ^d	3 x 10 ^{1e}	NV	NV	NV	NV
2	8 x 10 ¹	1 x 10 ²	3 x 10 ²	4 x 10 ²	2 x 10 ²	4 x 10 ²
3	5 x 10 ¹	ND ^f	6 x 10 ²	1 x 10 ³	4 x 10 ²	6 x 10 ²
4	3 x 10 ²	4 x 10 ²	3 x 10 ³	3 x 10 ³	2 x 10 ³	2 x 10 ³
5	NV	NV	3 x 10 ²	2 x 10 ²	4 x 10 ²	2 x 10 ²
6	8 x 10 ¹	1 x 10 ²	2 x 10 ³	2 x 10 ³	1 x 10 ³	2 x 10 ³
7	3 x 10 ¹	5 x 10 ¹	2 x 10 ³	1 x 10 ³	9 x 10 ²	1 x 10 ³

^aTemperature at which aliquots of homogenates of pooled internal organs from striped bass were stored for 48 hours prior to being assayed for virus by the plaque method.

^bAliquots of striped bass tissue homogenates were stored in sterile glass vials.

^cAliquots of tissue homogenates were stored in sterile plastic bags.

^dNo IPNV was detected.

^ePlaque forming units of IPNV per ml of homogenate.

^fNot done.

Table 15: Recovery of infectious pancreatic necrosis virus (IPNV) from IPNV-infected striped bass stored whole.

STORAGE TEMP(°C) ^a	LENGTH OF STORAGE		
	0 DAYS	2 DAYS	14 DAYS
	9 / 10 ^b		
	(10 ² -10 ⁵) ^c		
4		10 / 11	3 / 3
		(10 ² -10 ⁴)	(10 ³ -10 ⁴)
-20		4 / 13	1 / 3
		(10 ² -10 ³)	(10 ³)
-70		0 / 17	0 / 3
		(NV ^d)	(NV)

^aTemperature at which IPNV-infected striped bass fingerlings were stored intact in plastic bags.

^bNumber of fish that were positive for IPNV per number of fish that were assayed for virus by the plaque method.

^cRange of IPNV titer expressed as plaque forming units per gram of pooled internal organs.

^dNo IPNV was recovered.

carrier striped bass that were stored intact in the refrigerator at 4°C. All virus infectivity was lost in IPNV-carrier striped bass that were stored at -70°C (Table 15). The loss was evident after only 48 hours of storage. In fish that were stored at -20°C, loss of infectivity was intermediate between that observed at 4°C and -70°C.

Comparison of IPNV Isolates

Protein Electrophoretic Patterns

Three IPNV isolates, one from striped bass (IPNV-Sb), one from Atlantic menhaden (IPNV-M), and the North American reference salmonid isolate (VR-299), were purified over discontinuous CsCl gradients. Viral proteins were analysed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Similar protein profiles were demonstrated (Figure 5). The relative mobility (D_f) of each viral polypeptide and molecular weight standard was determined by dividing the actual distance traveled by each protein band by the distance moved by the dye front. A standard curve was developed by plotting the D_f of each molecular weight standard against the \log_{10} of its molecular weight. The molecular weight of each viral protein in the polyacrylamide gel was determined from the standard curve. For IPNV-Sb and IPNV-M, there were polypeptide bands corresponding to molecular weights of 95, 53, 51, 31, and 29 K. For VR-299, there were proteins with molecular weights corresponding to 95, 53, 51, and 29 K.

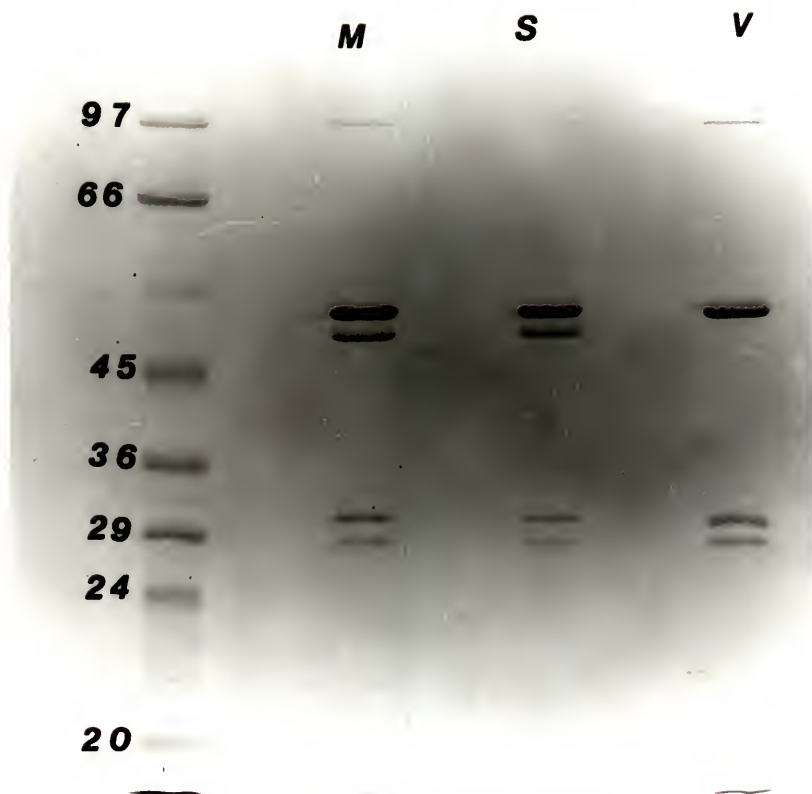


Figure 5: Electrophoretic profile of polypeptides from three isolates of infectious pancreatic necrosis virus (IPNV) fractionated on a discontinuous 10% polyacrylamide gel. The three IPNV isolates are: striped bass (S), menhaden (M), and the North American VR-299 (V). The left lane contains the following molecular weight markers: phosphorylase b (97,400), bovine albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,100). Numbers on the gel indicate molecular weights $\times 10^3$. The lowest band represents the dye front.

Neutralization Kinetics

Neutralization kinetics reveals the pattern and rate at which virus becomes neutralized in the presence of excess antibody. Each of three IPNV isolates (IPNV-Sb, IPNV-M, and VR-299) was reacted with homologous and heterologous antibody, and the residual infectivity at several time points was measured. The neutralization kinetic curves for the three IPNV isolates were similar for homologous and heterologous antibody reactions (Figure 6). The rate of neutralization (K) was calculated using the formula $K = D/t \cdot 2.3 \log V_0 / V_t$, where D = reciprocal of the dilution of antibody, t = 0.25 minutes, V_0 = total virus, and V_t = number of virus plaques at 0.25 minutes (Macdonald & Gower, 1981). The calculated neutralization rates (K) were of the same magnitude for most combinations of IPNV isolates and antibodies (Table 16). The only exception was the increased rate detected for the reaction of VR-299 with its homologous antibody.

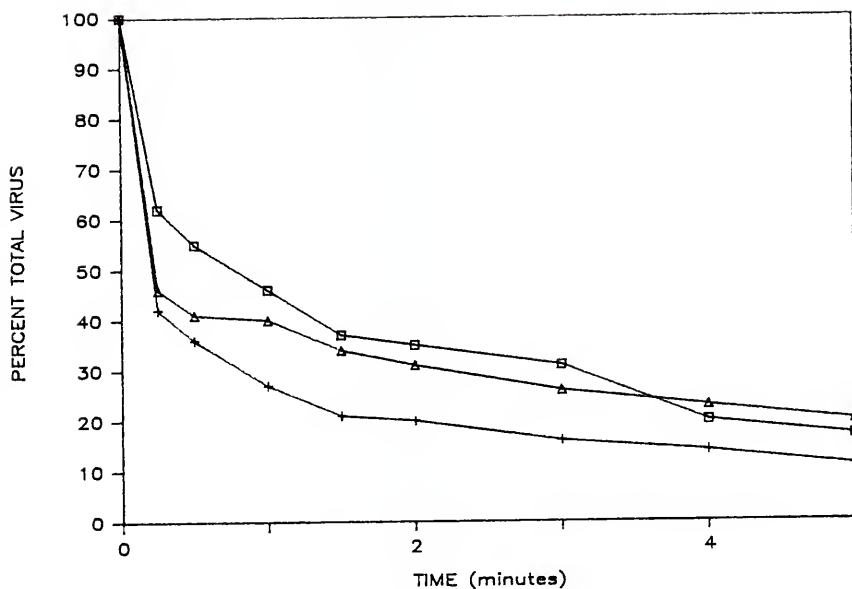


Figure 6 A. The IPNV isolates were tested with antibody against the striped bass IPNV isolate.

Figure 6: Comparison of the neutralization kinetics of three isolates of infectious pancreatic necrosis virus (IPNV); striped bass (□), menhaden (+) and the North American isolate VR-299 (Δ). Equal volumes of diluted antibody and virus were mixed, incubated at 4°C, and sampled at the indicated times. Residual infectivity at each time point was determined by the plaque method, and expressed as the percentage of total number of IPNV plaques.

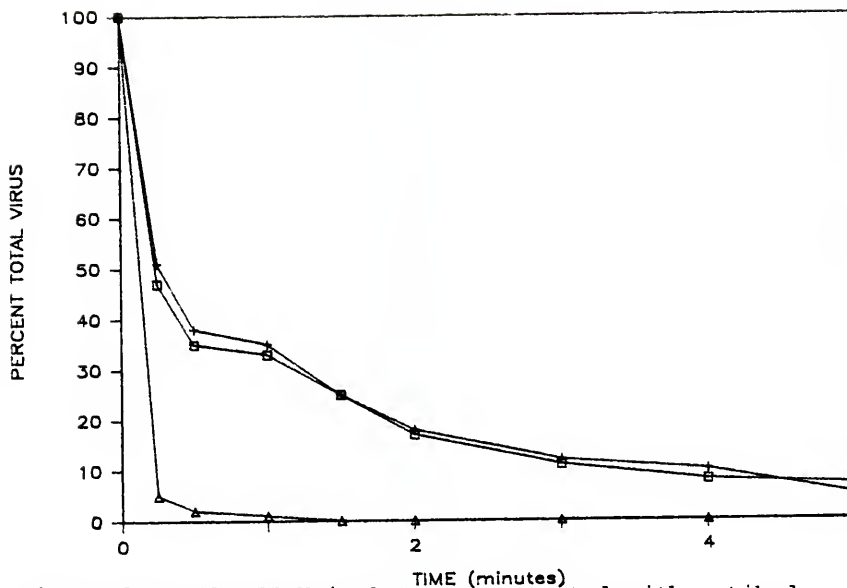


Figure 6 B. The IPNV isolates were tested with antibody against the North American isolate VR-299.

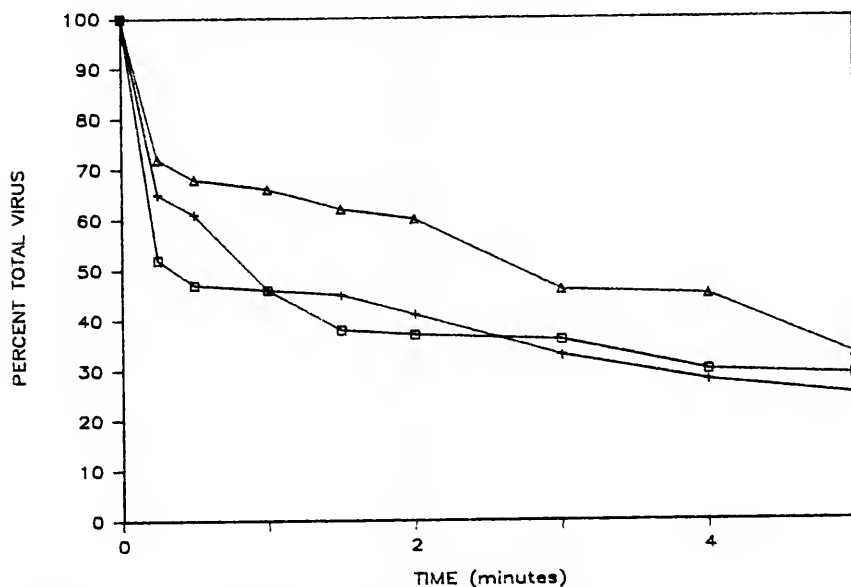


Figure 6 C. The IPNV isolates were tested with antibody against the menhaden IPNV isolate.

Table 16: Neutralization rates for three infectious pancreatic necrosis virus (IPNV) isolates reacted with homologous and heterologous antisera.

ANTISERUM	Virus ^a		
	IPNV-M ^b	IPNV-Sb ^c	VR-299 ^d
IPNV-M	3×10^5 ^e	7×10^5	5×10^5
IPNV-Sb	9×10^5	4×10^5	4×10^5
VR-299	9×10^5	6×10^5	24×10^5

^aEach IPNV isolate was reacted individually with rabbit antiserum against each of the isolates. Total virus and residual infectivity at 0.25 minutes were determined by plaque assay.

^bThe menhaden isolate of IPNV.

^cThe striped bass isolate of IPNV.

^dThe standard North American isolate of IPNV.

^e The rate of neutralization was assumed to be linear for the first 0.25 minutes of the reaction between antibody and virus. The neutralization rate (K) was calculated for each trial using the formula $K = D/t \times 2.3 \times \log V_0 / V_t$, where D = dilution of the antiserum, t = 0.25 minutes, V_0 = total number of viral plaques, and V_t = number of viral plaques at 0.25 minutes.

CHAPTER FOUR DISCUSSION

Infectious pancreatic necrosis virus was isolated from moribund striped bass fry in a hatchery on the Chesapeake Bay (MD) (Schutz et al., 1984). Efforts to rear striped bass in hatcheries have increased recently (Schutz et al., 1984), partly because numbers of striped bass in the Chesapeake Bay have been declining (Goodyear et al., 1985). The reasons for the observed decline are not known. It is known, however, that IPNV virus causes significant losses in salmonids raised in hatcheries (Wolf et al., 1960) and is pathogenic for Atlantic menhaden in Chesapeake Bay (Stephens et al., 1980). The current study was initiated to investigate what effects IPNV infection has on striped bass, how IPNV can be transmitted, and whether the IPNV recovered from striped bass is related to the IPNV isolate from menhaden.

In IPNV infection trials using 1- to 20-day striped bass, mortalities in different strains of striped bass challenged with water borne IPNV were not higher than in controls. Efforts were made to duplicate the conditions that existed when IPNV was originally isolated from striped bass (Schutz et al., 1984). However, none of the clinical signs or histopathological lesions described by Schutz et

al. (1984) were observed in the experimental striped bass. The etiology of the mortalities and histological abnormalities described by Schutz et al. (1984) is not known.

Peaks of mortality in IPNV-challenged and control fish coincided within trials but were not predictable between trials. The reasons for the deaths are not known. Possibly contaminants (e.g. bacteria, ammonia) introduced with the brine shrimp nauplii fed to the fish may have accounted for the mortality pattern.

It is clear, however, that immersion exposure to virus did not cause predictable mortality in striped bass, even in IPNV-challenged fish subjected to an abrupt pH change. In contrast, young brook trout showed increased mortality after immersion challenge with the striped bass isolate of IPNV (P. E. McAllister, National Fish Health Research Laboratory, Kearneysville, WV; unpublished data). The reasons for the difference in IPNV pathogenicity in fishes are not known.

Striped bass demonstrated age-related differences in susceptibility to IPNV infection after waterborne challenge. Three weeks after immersion IPNV challenge, only striped bass that had been exposed at one day post-hatch, contained virus. No virus was recovered from striped bass that were exposed at 26 days or older to water borne IPNV. Explanations for these findings probably involve the nature of the integument in very young fish,

and the speed with which effective defense mechanisms develop in these fish. The external integument of newly hatched fry performs exchange functions that are later performed by the gills and other organ systems (Johansen, 1982; Roberts et al., 1973). Possibly the immature integument might provide a site to which exogenous virus can attach, enter and multiply--a site that later becomes inaccessible to virus. In addition to physical changes in the integument, fish may quickly develop other nonspecific defense mechanisms such as inteferon and cellular defense systems, that may protect fish from waterborne microorganisms (de Kinkelin & Dorson, 1973; Manning et al., 1982; Tatner & Manning, 1985). A specific humoral response probably is not a major factor in protecting very young fry (Manning et al., 1982; Manning & Mughal, 1985). None of the experimental striped bass immersed in IPNV developed virus-neutralizing antibodies.

In contrast to the lack of infectivity of IPNV in all but the youngest striped bass exposed to waterborne IPNV, experimental inoculation of IPNV into striped bass resulted in asymptomatic carriers that contained infectious virus for longer than one year. No overt signs of disease, such as "spinning" or increased mortality, were seen in virus infected striped bass, even in IPNV-injected striped bass that were subjected to environmental stress. Similarly, no histopathology was detected in experimental IPNV-infected

striped bass. Atlantic salmon, Salmo salar, develop subclinical IPNV infections like striped bass; however, unlike striped bass, Atlantic salmon do develop degenerative pancreatic lesions (Swanson & Gillespie, 1979). The significance of the IPNV-induced histological lesions is not known.

Striped bass did become infected with IPNV after ingesting IPNV-carrier brook trout. Like brook trout, menhaden are susceptible to IPNV-induced disease (Stephens et al., 1980). The virus can be isolated from menhaden during their annual spring epizootic in the Chesapeake Bay (Stephens et al., 1980). Possibly striped bass may be exposed to IPNV by consuming IPNV-infected menhaden. The source of IPNV infection of menhaden in Chesapeake Bay has not been reported, but brook trout, as well as other fish, probably can become infected with IPNV via the sex products (Wolf & al., 1963; Bullock et al., 1976; Seeley et al., 1977; Dorson & Torchy, 1985).

Experimental transmission studies did not demonstrate the spread of IPNV from striped bass sex products to offspring. Virus was not recovered from the milt or urine of survivors in the population of striped bass from which the original IPNV isolate was obtained. In addition, IPNV was not isolated from the offspring from experimentally infected striped bass adults or from offspring of IPNV-exposed sex products. The virus was isolated from striped bass sperm and larvae collected from the Chesapeake Bay in

1984, but not in 1985 or 1986 (F. M. Hetrick, University of Maryland, unpublished data). The significance of these findings is unclear.

Although no virus was recovered from any striped bass organs sampled from Chesapeake Bay, virus-neutralizing antibody was found in the older fish caught in the winter of 1984 and young-of-the-year fish sampled in the summer of 1985. Possibly the older fish were exposed to the virus during the spring IPNV-epizootics in menhaden. Results from neutralization kinetics and SDS-PAGE of viral proteins demonstrate the close relationship between IPNV isolated from striped bass (IPNV-Sb) and from menhaden (IPNV-M).

Neutralization kinetics are sensitive tests for comparing antigenic relatedness between viruses (Ashe & Scherp, 1963) and have been used to categorize IPNV-isolates into distinct serotypes (Macdonald & Gower, 1981). In the current study, the neutralization curves and the rates of neutralization of the striped bass isolate of IPNV (IPNV-Sb) were virtually identical to those of the menhaden isolate (IPNV-M). Use of the same analytical technique revealed that both isolates are closely related to the standard North American isolate (VR-299). The neutralization rate of the reaction of the VR-299 isolate with its homologous antibody was one magnitude higher than rates determined for the other neutralization reactions. This probably indicates that

the antiserum either recognized or was more avidly bound by some antigenic determinant on VR-299 that was not present on the other two isolates tested. However, in all other neutralization reactions, VR-299 patterns were like those for IPNV-Sb and IPNV-M, indicating a close relationship between the three isolates.

In addition, polyacrylamide gel electrophoresis of the three IPNV isolates demonstrated similar viral polypeptide bands. The calculated molecular weight of proteins of IPNV-Sb and IPNV-M were 95000, 53000, 51000, 31000 and 29000. All but one (51000) protein band were demonstrated for the North American isolate (VR-299). The molecular weights of the viral proteins of IPNV-M and VR-299 have been reported to be 86000, 56000, 30000, and 27000 (Stephens, 1981; Stephens & Hetrick, 1983). The actual molecular weights attributed to the viral polypeptides has varied, even within the same laboratory (Dobos, 1977; Dobos & Rowe, 1977; Dobos et al., 1977). The variation probably is related to differences in the experimental protocols used (Dobos & Rowe, 1977). For purposes of the present study, the important finding is the demonstrated similarity between the menhaden and the striped bass isolates of IPNV.

As previously discussed, striped bass did not become infected with IPNV after waterborne challenge, but became inapparent IPNV-carriers after inoculation or ingestion of IPNV. Virus-infected striped bass did shed sufficient IPNV

to infect brook trout that were located in tanks downstream from the striped bass. Previous reports have implicated IPNV-infected trout as the source of IPNV infection of nonsalmonids, such as Catostomus commersoni (Sonstegard et al., 1972). The present results demonstrate that IPNV can be transmitted from a nonsalmonid species to trout.

The spread of IPNV from healthy appearing (both grossly and histologically) striped bass to a susceptible fish species has practical implications. If IPNV-carrier striped bass are transported to areas that were previously IPNV-free, the striped bass pose a potential threat to fish species in the watershed. The virus is relatively stable in the environment, remaining infective for months in aqueous environments (Tu et al., 1975; Toranzo & Hetrick, 1982). Therefore, testing a population of striped bass for IPNV prior to introduction into IPNV-free areas would seem advisable. A series of experiments were performed to determine what samples should be taken and how the samples should be handled to improve the recovery of IPNV from virus-infected striped bass.

Virus was reisolated from IPNV-infected striped bass most often from anterior kidney and from the spleen, but never from the brain. A similar pattern of IPNV recovery is found in trout (Wolf & Quimby, 1969; Yamamoto, 1974). These results differ from those of Dorson (1982) who

contended that the brain of trout IPNV-carriers may be the only tissue containing IPNV.

When IPNV was detected in blood samples from striped bass, the virus was found associated with the leucocytes. Only occasionally was IPNV recovered from plasma. Swanson and Gillespie (1982) reported similar findings from IPNV-infected brook and rainbow trout. Swanson and Gillespie (1982) separated the blood cells over a Ficoll gradient prior to virus assay. The current study developed a simple separation procedure consisting of removal of the buffy coat from centrifuged hematocrit tubes. This procedure permitted virus assay of smaller blood volumes than have been reported previously (Swanson & Gillespie, 1982; Yu et al., 1982). To detect levels of virus lower than those recovered in this study white blood cells can be cocultured with virus-susceptible cells (Yu et al, 1982). Another sensitive assay permits recovery of IPNV from the supernatant fluid from mitogen stimulated lymphocytes from IPNV-infected Atlantic salmon (Knott and Munro, 1986). Whatever procedure is utilized to isolate IPNV from blood and blood components, the samples should be assayed as quickly as possible (Swanson & Gillespie, 1982).

Previous investigations on the effects of storage of samples on IPNV recovery have used virus-containing culture fluids, or tissue homogenates to which IPNV was added (Malsberger and Cerini, 1963; Wolf, 1964; Wolf et al., 1969; McMichael et al., 1975). Use of liquid samples

permits obtaining initial levels of infective virus in the samples. Data from stored IPNV-infected striped bass tissue homogenates were similar to those reported by the other authors. An increased loss of virus titer was observed in homogenate samples stored at 4°C, compared to values obtained from samples stored at -20° or -70°C. Therefore, for best virus recovery, striped bass tissue homogenates should be stored frozen (-20 or -70°C).

In contrast, data from trials storing whole IPNV-infected striped bass fingerlings gave different results. All viral infectivity was lost in fish samples stored at -70° but was retained at 4°C. Retention of virus infectivity was intermediate in samples stored at -20°C. The retention of viral infectivity in whole striped bass stored at 4°C and loss of infectivity in fish stored at -70°C was somewhat unexpected. The disadvantage of using whole fish is that an initial virus titer can not be obtained. However, because there was a high (> 90%) incidence of IPNV-carriers in the experimental striped bass used in this study, the lack of virus infectivity in fish stored at -70°C must be a result of events associated with storage at the lower temperatures. Perhaps the different rates at which freezing and thawing occurs in whole fish compared to aqueous solutions may affect viral infectivity.

The observed differences of IPNV stability in stored whole fish and in stored homogenates were not due to a

difference in storage containers. When IPNV-infected tissue homogenates were stored in plastic bags similar to those in which whole fish were stored, homogenate samples again lost infective virus at 4°C, but IPNV remained infective when stored frozen (-20°C and -70°C). The reasons for the variation in IPNV infectivity from intact and homogenized fish tissues are not known. However, demonstration of the variation emphasizes the importance of experiments that attempt to replicate field conditions.

Previous investigations that utilized IPNV-infected cell cultures or tissue homogenates may, or may not, accurately reflect practical field conditions. Different IPNV isolates vary in stabilities during storage (Dorson et al., 1978; McMichael et al., 1975; Malsberger & Cerini, 1963). Further studies are needed to determine if the lability of various IPNV isolates is similar to that demonstrated for the striped bass isolate of IPNV in stored striped bass samples. Current data demonstrate that for detection of IPNV-Sb infectivity in striped bass, samples should be stored intact at 4°C and assayed within two weeks.

Bullock and Stuckey (1975) reported that steroids increase the recovery of infectious agents from inapparent trout carriers. Increased detection of IPNV was observed in IPNV-inoculated fish that were given an intraperitoneal injection of steroid fifteen months after IPNV injection.

Apparently the steroids temporarily reversed the observed decline of virus titers in IPNV-infected striped bass.

Tissue titers in IPNV-inoculated striped bass remained relatively constant over the first 10 days. In contrast, IPNV levels peak at three days after IPNV inoculation in Atlantic salmon (Swanson & Gillespie, 1979), and in rainbow trout that are susceptible to IPNV-induced mortality, IPNV titers reach high titers at seven days after exposure (Okamoto et al., 1984). Although Yamamoto (1975b) suggested a correlation between virus-neutralizing antibody and IPNV titers in trout, no such relationship was observed in striped bass. Antibody was not detected in IPNV-injected striped bass until seven days post inoculation and reached peak values at approximately three weeks. During this same time period, levels of virus recovered from IPNV-inoculated striped bass remained uniform, apparently unaffected by the presence of the antibody. In fact, virus titers remained uniform during the first two months after IPNV-injection and gradually declined over a 15 month period.

The virus was frequently recovered from the anterior kidney, spleen, and leucocytes--tissues that are immunologically active in fish (Ellis, 1982). The effects of IPNV on the immune system of fish are just beginning to be investigated. For instance, Knott and Munro (1986) reported that lymphocytes from IPNV-infected Atlantic salmon demonstrate decreased mitogen activity. Work with

another birnavirus, infectious bursal disease virus (IBDV) in chickens, has shown that IBDV is directly immunosuppressive (Faragher et al., 1972), and bursal (B) lymphocytes are the target cells for the virus (Hirai & Calnek, 1979). Because a bursa-equivalent has not been identified for fish, demonstration of IPNV-induced immunomodulation in fish will probably be more difficult to elucidate.

Depression of the humoral response of striped bass to IPNV was not observed in the current study. In fact, IPNV was strongly antigenic to striped bass and induced significant antibody titers after IPNV-injection. A rise in antibody levels was detected in IPNV-injected striped bass at 7 days post injection after both a primary and secondary inoculation. In a classical anamnestic response, antibody levels rise faster in the secondary response (Eisen, 1980). The controversy over whether fish demonstrate a true anamnestic response has been reviewed (Dorson, 1984). In the current experiments, striped bass did not appear to exhibit an anamnestic response but they did mount a humoral response to IPNV infection.

Immersion in IPNV, however, was not sufficient to stimulate detectable levels of virus-neutralizing antibody. Even in virus-carrier striped bass that were given a second IPNV exposure by immersion, antibody levels did not increase after the second challenge.

Chronic IPNV-carrier striped bass that received exogenous steroid did not demonstrate any change in the titers of virus-neutralizing antibody. Administration of steroids prior to IPNV injection, did delay and reduce the humoral response of striped bass. Steroid induced immunosuppression has been described also in trout (Anderson et al., 1982). In addition, Anderson et al. (1982) reported that trout given a steroid dose of 200 mg/kg did not appear unhealthy during the 23 days of the experiment. Steroid, administered at a rate of 100 mg per kg body weight, was lethal to striped bass observed over a three month period. However, none of the IPNV-injected striped bass that were treated with steroids developed any clinical signs or histological lesions attributable to IPNV.

In conclusion, then, IPNV is not a major pathogen for striped bass, but striped bass can be inapparent IPNV-carriers. Virus-carriers may pose a potential threat to IPNV-susceptible fish species. Therefore, prior to transport of striped bass into IPNV-free areas, the striped bass should be tested for IPNV. However, isolation of IPNV from a population of striped bass should not be used as a reason to destroy the fish since striped bass apparently are resistant to IPNV-induced disease.

APPENDIX
SOURCES OF SUPPLIES AND EQUIPMENT

- Aldrich Chemical Corporation, Inc. (Milwaukee, WI)
Glycerol
- American Scientific Products (McGraw Park, IL)
Heparinized microhematocrit capillary tubes
- Ames Company (Elkhart, IN)
N,N,N',N' tetramethyl ethylenedianine (TEMED)
Coomassie blue
- Amicon Corporation (Danvers, MA)
Microconcentrators (CENTRICON)
- Armour Pharmaceutical (Tarrytown, NY)
Fetal bovine serum
- Beckman Instruments, Incorporated (Palo Alto, CA)
Cellulose nitrate centrifuge tubes (5/8" x 4")
Ultra-clear centrifuge tubes
Ultracentrifuge (Beckman L5-50B Ultracentrifuge)
- Becton, Dickinson & Co. (Rutherford, NJ)
Syringes and needles
- Bellco Glass Inc. (Vineland, NJ)
Multi-stir
- Bethesda Research Laboratory (Gaithersburg, MD)
Sucrose (ultrapure enzyme grade)
- Biorad (Richmond, CA)
Bis-acrylamide
- CGA Corporation (Chicago, IL)
Precision low temperature incubator
- Corning Glass Works (Corning, NY)
Tissue culture flasks and bottles
- Commercial Products Corporation (Manitowoc, WI)
Kelvinator Series 500 Freezer
- Crescent Research Chemicals (Paradise Valley, AZ)
Tricaine methanesulfonate (MS-222)

- Difco Laboratories (Detroit, MI)
Freunds' incomplete adjuvant
- DuPont Company (Wilmington, DE)
Trichlorotrifluoroethane (FREON)
- Fisher Scientific Company (Fairlawn, NJ)
Alundum (90-mesh)
Bovine albumin
Cesium chloride
Crystal violet
Disodium ethylenediamine tetraacetate (EDTA)
Ethanol
Formalin
Glacial acetic acid
Methanol
Phenol reagent (Folin Ciocalteu Reagent)
Plastic bags (WHIRL-PAC)
Sodium bicarbonate
Sodium carbonate
Sodium hydroxide
Sodium phosphate
Sodium potassium tartrate
- Flow Laboratories (McLean, VA)
Eagle's minimal essential medium
Normal calf serum
96-well tissue culture plates
- FMC Corporation (Rockville, ME)
Agarose
- Gelman (Ann Arbor, MI)
Membrane filters (ACRODISC)
- GIBCO (Grand Island, NY)
Trypsin
- Harleco (Philadelphia, PA)
Bromophenol blue
- Heath Company (Benton Harbor, MI)
Regulated HV Power Supply
- Hewlett-Packard (Ft. Collins, CO)
Series 9800 Desktop computer
- International Equipment Company (Needham Heights, MS)
IEC Centra-7R Centrifuge
International micro-capillary centrifuge
- LKB-Produkter AB (Brooma, Sweden)
Hydroxyethyl methacrylate (HISTORESIN)

Miles Laboratories (Naperville, IL)
Eight-well culture plates

Nutrition Biochemical Corporation (Cleveland, OH)
Glycine, aminoacetic

Sigma Chemical Company (St. Louis, MO)
Acrylamide
2-Mercaptoethanol (2-ME)
Molecular weight markers
Penicillin
Polyethylene glycol
Sodium chloride
Sodium lauryl sulfate (SDS)
Streptomycin
Trizma base
Trizma hydrochloride

Sorvall (Ivan) Incorporated (Newtown, CT)
Sorval RC2-B Centrifuge

Squibb (E. R.) and Sons, Inc. (Princeton, NJ)
Triamcinolone acetonide (Kenalog-40)

Thomas (Arthur H.) Company (Philadelphia, PA)
Dialysis tubing (1/4"; Thomas tubing)

Varian Associates Inc. Instrument Group (Palo Alto, CA)
Spectrophotometer (Cary 219)

Virtis Company (Gardiner, NY)
VirTis "23" homogenizer

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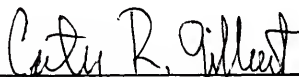
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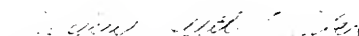
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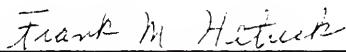
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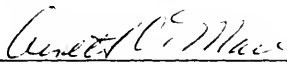
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